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CORRECTION

On page 255, line 15, Vol. 185, No. 1, July, 1950, read *Heating with 1 ml. of 90 per cent (by weight) du Pont sulfuric acid* for *Heating with 90 per cent (by weight) du Pont sulfuric acid*.

DISSYMMETRY IN NUCLEOTIDE SEQUENCE OF DESOXYPENTOSE NUCLEIC ACIDS*

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(Received for publication, June 21, 1950)

The discovery that the composition of the desoxypentose nucleic acids deviated very considerably from that required by the "tetranucleotide" hypothesis (2, 3) and that in certain cases nucleic acids derived from different species could be shown to differ in composition (2-5) made the study of the sequence in which the component nucleotides are aligned in a nucleic acid particularly attractive. The first orienting approach to this problem appeared to lie in the decision as to whether the DNA¹ of calf thymus was generally uniform throughout the molecule, *i.e.* whether it showed a recognizable regularity or periodicity of composition, or whether certain flaws could be demonstrated in its structure, indicating a more complex pattern of the distribution of purine and pyrimidine nucleotides. The latter has now been shown to be the case.

EXPERIMENTAL

Material

Substrates—All DNA preparations were highly polymerized. The specimen from yeast, Y-DNA, was prepared by the procedure described before (9). Its DNA content, when assayed against a standard sample of T-DNA (2), corresponded to 92.5 per cent. Its composition in respect to purines and pyrimidines, which is reported below, was in good agreement with a previous analysis of another preparation (3). It contained less than 2 per cent of PNA. Two preparations from calf thymus were employed. T-DNA, Preparation 1, has been described previously as Preparation 3 (2). Another specimen, T-DNA, Preparation 2, was isolated by essentially similar procedures, though with several minor modifications.

* This work has been supported by a research grant from the National Institutes of Health, United States Public Health Service. Some of its results have been reported in preliminary form (1).

¹ The following abbreviations will be used: DNA, desoxypentose nucleic acid (T-DNA, preparations from calf thymus, Y-DNA from yeast); PNA, pentose nucleic acid; C-DNase, crystalline desoxyribonuclease (6, 7); P-DNase, purified desoxyribonuclease (8); RNase, crystalline ribonuclease.

The ground tissue was washed twice with about 4 volumes of 0.1 M sodium citrate solution (pH 7.4), prior to the extraction of the nucleoprotein with 10 per cent aqueous NaCl. This procedure lowered the PNA content of the final preparation to less than 1.5 per cent.² The DNA preparation, depolymerized by alkali, which was employed in Experiment 4, was prepared from a 0.33 per cent aqueous solution of T-DNA, Preparation 1, which was cautiously adjusted to pH 12.5 by the addition of N sodium hydroxide at room temperature. In this manner, the initial viscosity of the solution (η_{sp}) was decreased from 64 to 3 (11). The solution was then carefully neutralized with N HCl.

Enzymes—C-DNase, prepared by the method of Kunitz (6, 7), was obtained from the Worthington Biochemical Laboratory, Freehold, New Jersey. For the preparations of P-DNase (8) and RNase, we are greatly indebted to Dr. M. McCarty and Dr. M. Kunitz, respectively.

Enzymatic Digestion

The action of DNase on DNA was studied in numerous experiments, designed to permit the analysis of dialyzable breakdown products and non-dialyzable remnants at different periods. In order to conserve space, the experimental conditions are summarized succinctly in Table I.

The experiments were carried out in closed bags made of cellulose casing (23/32 NoJax, Visking Corporation, Chicago) in which was placed the buffer solution of the substrate, made 0.003 M with respect to $MgSO_4$ and containing 0.001 per cent of ethyl mercurithiosalicylate and the indicated amounts of enzyme. In control experiments DNase was omitted. The volumes of solution in the dialysis bags are listed as "inside fluid" in Table I. Dialysis was carried out against the same buffer mixtures, or water, as indicated. When limited volumes of outside fluids were employed, they were replaced periodically; the total volumes used in each dialysis period are listed as "outside fluid" in Table I. In some experiments, the enzymatic digestion and dialysis were repeated following the addition of fresh enzyme. Aliquots of the inside and outside fluids were removed periodically for analysis and evaporated in the frozen state in a vacuum.

* It should be stressed that, wherever possible, the DNA preparations employed should contain no more than 2 to 3 per cent of contaminating PNA. In analytical studies of DNA composition this is essential because of the high guanine and cytosine contents of PNA from animal tissue (10). It becomes even more imperative in studies of the type reported here, since PNA originally present as a contaminant will assume a relatively large proportion in the dialysis residue resulting from the enzymatic digestion of DNA, unless it is removed as completely as possible, as has been done here, by treatment with RNase. The remaining small residue of PNA, resistant to degradation by RNase, is extremely rich in guanine (unpublished experiments with Dr. B. Magasanik), but, as it comprises only 2 to 4 per cent of the DNA core (1), this can normally be neglected.

Analytical Procedures

Estimation of Purines and Pyrimidines—The methods employed for the separation and quantitative determination of individual nitrogenous components, in both the intact nucleic acids and the enzymatically produced fragments, were essentially based on the microprocedures previously published from this laboratory (12, 13, 2). Each value reported here represents the average of six to sixteen individual determinations. Several modifications permitted the use of even smaller quantities than before and simplified the determination of the pyrimidines.

By the determination of total P in all hydrolysates of nucleic acids or polynucleotides subjected to chromatography, it became possible to determine the molar ratios of individual nitrogenous constituents to phosphorus, even when the small amount of material available precluded the adjustment of the hydrolysate to an exact volume (13). All desired proportions could then be computed from these ratios. For the estimation of the purines, about 2 mg. of DNA were used (0.3 cc. of $N H_2SO_4$, 1 hour at 100° , sealed tube), and for the determination of the pyrimidines, 6 to 8 mg. of DNA (0.3 cc. of concentrated formic acid, 2 hours at 175° , sealed tube, following the removal of purine hydrochlorides (13) in 0.3 cc. of methanol-HCl).³ The position of the separated components on the chromatogram was determined with the aid of the Mineralight ultraviolet lamp (14-16).

When all principal nitrogenous constituents were to be determined on one sample of the order of 2 mg., use was made of an as yet unpublished modification developed in this laboratory.⁴ In this procedure, which was employed in Experiments 5 to 7, about 2 mg. of DNA or its degradation products were heated in a sealed tube with 0.2 cc. of concentrated formic acid for 2 hours at 175° . Aliquots of the hydrolysate served for P determination and for the chromatographic separation on filter paper and spectroscopic estimation in the usual manner (12) of guanine, cytosine, adenine, and thymine (aligned in this descending order) by means of a solvent system consisting of 6 parts of *n*-butanol and 1 part of 0.6 to 0.7 *N* aqueous ammonia.

Miscellaneous Estimations—*Purine-bound desoxyribose* was determined colorimetrically by means of the reaction with diphenylamine (17), with a highly purified sample of T-DNA serving as the reference standard (2). The *DNA contents* were determined from these figures, when account was taken of the proportions of purines to pyrimidines found in a given sample

³ Correction should be made of a misprint on page 718 of a previous publication (13). The bomb tubes used had the following dimensions: 220 mm. long, inside diameter 6.5 mm., outside diameter 8.5 mm.

⁴ Unpublished experiments with Mrs. C. Green and Dr. M. E. Hodes.

TABLE I
Enzymatic Digestion and Dialysis

Ex- peri- ment No.	Substrate		Digestion 1			Digestion 2		
	Material	Quan- tity	Enzyme	Inside fluid*	Outside fluid†	Enzyme	Inside fluid*	Outside fluid†
1	Y-DNA	mg. 10	0.35 P-DNase	cc. 1	20 cc. b. (24 hrs.)	mg. 0.7 RNase	cc. 9	1000 cc. b. (24 hrs.), r. w. (24 hrs.)
2	T-DNA, Prepa- ration 1	102.5	3.5 P-DNase	8	200 cc. b. (24 hrs.)	0.5 C-DNase	9	1000 cc. b. (24 hrs.), r. w. (62 hrs.)
3†	"	101	0.5 C-DNase	8	200 " (24 ")			
4	Same, alkali-de- polymerized	6.3	0.5 "	2.7	1000 cc. b. (24 hrs.), r. w. (48 hrs.)	0.5 C-DNase, 0.5 RNase	22	1000 cc. b. (11 hrs.), r. w. (44 hrs.)
5	T-DNA, Prepa- ration 2	250	0.5 "	20	600 cc. b. (24 hrs.)	0.5 C-DNase	5	150 cc. b. (7 hrs.), 100 cc. w. (17 hrs.), 75 cc. b. (18 hrs.), 100 cc. w. (8 hrs.)
6	"	250	0.5 " 0.5 RNase	15	1600 cc. b. (23 hrs.), r. w. (29 hrs.)	0.5 C-DNase, 0.85 RNase	41	1500 cc. b. (33 hrs.), 5400 cc. b. (39 hrs.)
7	"	500	0.6 C-DNase	20	400 cc. w. (4½ hrs.), 200 cc. b. (1½ hrs.), 1000 cc. w. (19 hrs.), 300 cc. b. (1 hr.), 1000 cc. w. (23 hrs.)			
8a	"	30	1.0 "	5	225 cc. b. (24 hrs.)			
8b	"	30	1.0 "	5	400 " w. (4 hrs.)			
8c‡	24.8% core	30	1.0 "	5	225 " b. (24 hrs.)			
8d‡	9% core	30	1.0 "	5	225 " " (19 hrs.)			
8e‡	9% "	30	1.0 "	5	225 " w. (23 hrs.)			

* In all experiments 0.02 M borate buffer was employed, with the exception of Experiment 5 in which 0.025 M glycylglycine buffer was used; the pH was 7.4 to 7.75. The following abbreviations are used: b., distilled water; w., buffer; w., distilled water; and r. w., running water.

† The duration of the dialysis is indicated in parentheses. The temperature of the experiments was 37° when the buffer solutions, specified in the preceding foot-note, were employed; 2-7° when distilled water served as the outside fluid. In both cases agitation by means of a mechanical rocker or vibrator was applied. The volumes indicate the total quantity of fluid used in each dialysis period.

‡ In Experiment 3, Digestion 2 was followed by Digestion 3 in which 0.6 mg. of crystalline DNase and 0.5 mg. of RNase were used. The volume of inside fluid was 9.5 cc.; as outside fluid, 1000 cc. of buffer (24 hours) and 800 cc. of water (24 hours) were used.

§ Per cent core is used to designate the dialysis residue (expressed as per cent of the original DNA) that remained after partial degradation with DNase and served as the substrate in these experiments.

by the procedures outlined in the preceding section; additional independent estimations were based on the total P content. *Phosphorus* (inorganic and total) was determined colorimetrically (18). *Purine-bound pentose* was estimated by means of the orcinol reaction (19), correction being made for the color produced with orcinol by DNA, and by the reaction with cysteine (20). The *PNA contents* were estimated from these figures. *Free ammonia* was determined by means of a microprocedure (21). The assay for the presence of *mononucleotides* in the dialysates was carried out by the chromatographic methods recently applied to ribonucleotides (16).

*Physical Measurements*⁵—The estimations of the *molecular weight* of the dialysis residues (Experiments 5 and 6) were performed by means of an

TABLE II

Dialysis Residue (As Per Cent of Original) Following Enzymatic Digestion of DNA

Digestion, hrs.	6	24	24	48	72
Dialysis, * hrs.	6	24	72	88	158
Per cent of original					
Experiment 1†. Y-DNA	42	5			
“ 2.. } T-DNA, Preparation 1	47	10	7		
“ 3... }	48	9		3.6	1.4
Control†..... }	99.4				
Experiment 4... } “ “ 1, alkali-			12		
Control†. . . . }			101		

* These figures include the periods during which digestion and dialysis were simultaneous.

† The experiments are numbered as in Table I.

‡ In the control experiments the enzymes indicated in Table I were omitted.

air-driven vacuum ultracentrifuge; 0.7 per cent solutions in 0.2 M NaCl were used. The *electrophoretic behavior* of the dialysis residue (Experiment 7) was studied in a Tiselius cell at 1.5°, with a 1 per cent solution in 0.02 M phosphate buffer of pH 7.4 (containing 0.15 M NaCl).

Results

Course of Enzymatic Digestion—The most obvious effects of the action of DNase on DNA are presented in Table II (Experiments 1 to 4) and Figs. 1 and 2 (Experiments 5 to 7 and 8 respectively). In control experiments

⁵ We are greatly indebted to Dr. M. MacKenzie and Dr. S. J. Circle for these determinations.

(without enzyme) or in experiments at low temperatures (Experiment 8b in Table I, and Fig. 2) practically no dialyzable fragments were found. At 37°, however, DNase soon brought about the disintegration of a major part of the original DNA to diffusible fragments. No essential differences were observed, in this respect, between purified (8) and crystalline (6, 7) DNase preparations from pancreas. These fragments, comprising 52 to

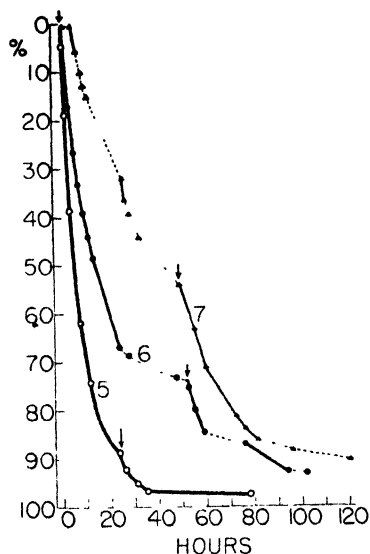


Fig. 1

Fig. 1. Course of degradation of T-DNA by C-DNase. The fractions transferred to the dialysate (in per cent of original DNA) are plotted as the ordinate; the abscissa indicates the duration of digestion or dialysis. The curves are numbered as the experiments in Table I. The solid lines represent simultaneous enzymatic digestion and dialysis (in the presence of buffer and Mg ion at 37°), the broken lines represent dialysis only (against water at 7°). The arrows indicate addition of fresh DNase.

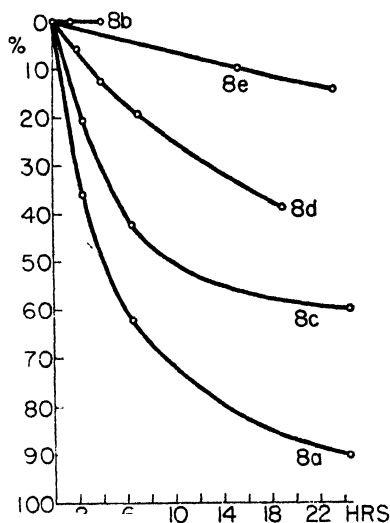


Fig. 2

Fig. 2. Comparison of the rates of enzymatic digestion of intact DNA and dialysis residues. Coordinates as in Fig. 1. The curves are numbered as the experiments in Table I.

53 per cent of the original DNA (Experiments 2 and 3), included almost no free ammonia or free phosphoric acid; less than 1 per cent of the amino N of the DNA and less than 2 per cent of its phosphorus were found as free ammonia and inorganic phosphate respectively. Similarly, mononucleotides could not be detected. Paper chromatograms of dialysates showed no discrete spots such as were found with ribonucleotides (16); instead, long streaks were observed that began at the starting point,

indicating the presence of many fragments of different size and composition.

The T-DNA preparation that had been subjected to depolymerization by alkali showed about the same degree of susceptibility to enzyme action as did the highly polymerized original material (Experiment 4). If, as has been suggested (22), the macromolecular structure of DNA is maintained by hydrogen bonds that are labile to acid or alkali, one would have to conclude that the degradative action of DNase, at least with respect to the production of dialyzable fragments, is not dependent upon the state of preservation of these bonds.

DNA Core—Even prolonged digestion with DNase failed to convert the entire DNA into readily dialyzable fragments (Table II and Fig. 1). The quantity of dialysis residue appears to decrease asymptotically with increasing duration of enzymatic digestion and dialysis. These residues have, on a previous occasion, been termed the DNA core (1). For the sake of conciseness, this term, when preceded by a percentage figure, will, in the following, denote a given dialysis residue, expressed as per cent of the original DNA remaining after the enzymatic action had been interrupted or had ceased. A similar procedure will apply to the dialysates.

The problem of the resistance of the core to enzymatic attack was studied in Experiment 8 (Fig. 2). Under comparable experimental conditions the 24.8 per cent core (Experiment 8c) furnished less dialyzable material than did the intact DNA (Experiment 8a); and the quantity of diffusible fragments released by the 9 per cent core was still smaller (Experiment 8d). If the liberation of dialyzable fragments is the direct result of DNase action, these experiments can be interpreted as showing that the core is more resistant to enzyme action than is DNA itself. Another noteworthy difference between the intact DNA and the core is revealed by a comparison of Experiments 8b and 8e in Fig. 2. In the presence of DNase, but under conditions unfavorable to enzymatic action (absence of Mg and buffer, low temperature), no dialyzable fractions were cleaved from the intact DNA, in contrast to the behavior of the 9 per cent core. It is not yet possible to decide between the following possibilities: (a) The action of DNase on the core continues, although more slowly, whereas the intact DNA is not acted upon by the enzyme; (b) a slow, non-enzymatic, spontaneous disintegration of the core takes place; (c) the core comprises a mixture of fragments of different sizes, some of which are sufficiently near the limit of permeability of the membrane to diffuse slowly.

Some evidence in favor of the last possibility could be gathered from experiments with the ultracentrifuge. The studies were carried out on a 24.8 per cent and a 2.5 per cent core. When the 24.8 per cent core was subjected to a centrifugal force of $172,000 \times g$ for 3 hours, the material

showed a slight tendency to separate from the meniscus; the heaviest portion contained in it may have had a molecular weight of the order of 10,000. No sedimentation whatever was observed in the 2.5 per cent core.

The electrophoretic study of a 10.4 per cent core showed it to exhibit one peak with a descending mobility of -12.4×10^{-5} cm.² volt⁻¹ sec.⁻¹. The addition to this solution of an equal amount of intact T-DNA produced two overlapping components with electrophoretic mobilities (descending) of -12.4 and -13.7 respectively.

TABLE III
Composition of Dialysate and Dialysis Residue

	Experiment 1,* Y-DNA		Experiment 2,* T-DNA, Preparation 1		
	Original fraction	58 per cent dialysate	Original fraction	53 per cent dialysate	7 per cent core
Moles per mole P					
Adenine	0.30	0.29	0.28	0.25	0.40
Guanine . .	0.18	0.19	0.22	0.21	0.19
Total purines .	0.48	0.48	0.50	0.46	0.59
Cytosine	0.15		0.19	0.21	0.10
Thymine	0.29		0.25	0.26	0.24
Total pyrimidines	0.44		0.44	0.47	0.34
Recovery .	0.92		0.94	0.93	0.93
Molar ratio					
Adenine to guanine . . .	1.7	1.5	1.3	1.2	2.1
Thymine to cytosine . . .	2.0		1.3	1.2	2.4
Adenine to cytosine . . .	2.0		1.5	1.2	4.0
Purines to pyrimidines . .	1.1		1.1	1.0	1.7

* The experiments are numbered as in Table I.

Composition of Enzymatic Digestion Products—The existence of a residue more resistant to enzymatic attack than the bulk of the material does not in itself permit conclusions to be drawn as to the uniformity of the latter. Such a residue could conceivably have originated at random from any part of a uniform DNA molecule, thus representing an accidental result of the enzymatic digestion process. This, however, does not seem to be the case, as will be shown by a systematic study of the composition of the degradation products.

Experiments 1 and 2, the results of which are summarized in Table III,

served for the first orientation. It can be seen that no very pronounced differences in composition were observed between the original DNA and the first portion of dialyzable breakdown products (comprising 53 to 58 per cent of the DNA), when analyzed as one fraction. It will be noticed, however, that the ratio of adenine to cytosine was lower in the dialysate (Experiment 2). Because of the relatively large quantities of DNA required in these experiments, only a few data could be collected on the DNA of yeast (Experiment 1); this substance seemed to resemble T-DNA in its behavior, as far as the adenine to guanine ratio is concerned. On the other hand, the analysis of the dialysis residue, studied in Experiment 2 as the 7 per cent core, revealed very significant divergences in the molar ratios of the individual purines and pyrimidines, as compared with the intact DNA. The proportions of adenine to guanine and especially to cytosine, of thymine to cytosine, and of purines to pyrimidines were increased very considerably, thus intensifying the dissymmetry in the distribution of constituents originally found in the intact T-DNA (2).⁶

A much clearer picture of this heterogeneity in the composition of T-DNA, as reflected in the changes in composition of its fragments liberated in the course of time by enzymatic action, is afforded by the more detailed Experiments 5 to 7, in which an attempt was made to reconstruct, by frequent and periodic analyses of dialysate and residue, the course of the disintegration of the DNA molecule. The combined results are presented graphically in Figs. 3 to 6.

The following conclusions may be drawn from these experiments: (a) None of the fragments, whether dialyzable or core, conforms in composition to a "tetranucleotide." Thus, the previous finding that intact DNA is far from a "statistical tetranucleotide" (2-5) can now be extended to the fragments obtainable by enzymatic digestion. (b) The composition of the dialyzable products (Curve I in Figs. 3 to 6) shows a characteristic trend as the digestion proceeds.⁷ The fragments most readily split off are relatively very rich in guanine and cytosine. The changes in different ratios differ in magnitude. Regardless of whether DNase is specific for the

⁶ Because of the disparity in size of the fractions, the divergent composition of a 7 per cent core cannot, of course, be expected to bring about a noticeable change in the composition of a dialysate comprising more than half of the original DNA.

⁷ In one experiment (Experiment 7) a brief digestion period with DNase (90 minutes) was followed by dialysis at 2° for 18 hours against several changes of distilled water, each of which was analyzed separately. The products which dialyze last under these conditions may be assumed to be the largest fragments produced during the initial digestion period. The analyses showed that the composition of these dialysates, which totaled 24 per cent of the original DNA, did not vary appreciably with the size of the fragments. The degradation by the enzyme does not appear to proceed haphazardly, but according to a plan inherent in the DNA molecule.

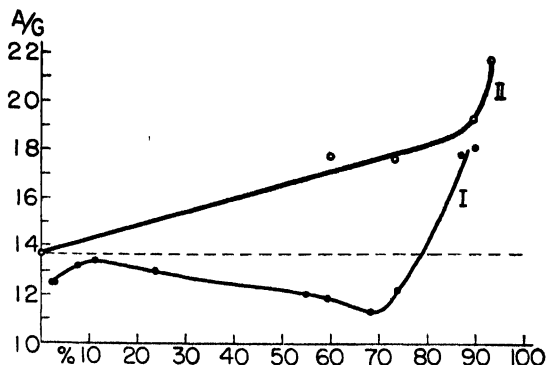


FIG. 3. Changes in the ratios of adenine to guanine in periodically collected portions of dialysate (Curve I) and in total dialysis residue (Curve II) in the course of enzymatic digestion and dialysis. The ratios are plotted as the ordinate; the abscissa indicates the degree of degradation of the DNA at the moment of sampling (expressed as per cent of original DNA found in total dialysate). The broken line represents the composition of the original DNA serving as substrate.

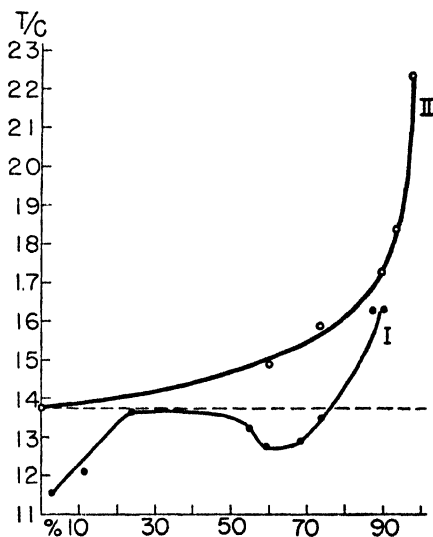


FIG. 4

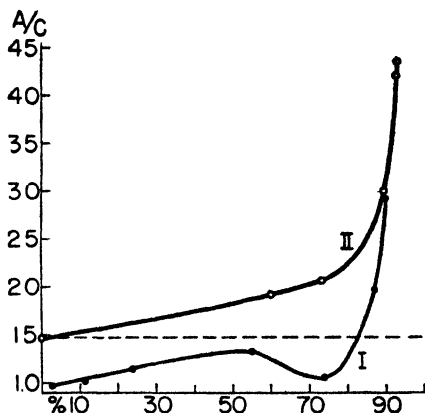


FIG. 5

FIG. 4. Changes in the ratios of thymine to cytosine in dialysate (Curve I) and dialysis residue (Curve II). See Fig. 3 for other explanations.

FIG. 5. Changes in the ratios of adenine to cytosine in dialysate (Curve I) and dialysis residue (Curve II). See Fig. 3 for other explanations.

cleavage of certain types of linkage, it is clear that the bulk of the DNA molecule itself is characterized by a marked lack of uniformity in the

distribution of individual purines and pyrimidines. (c) The relative concentration of pyrimidines is higher in the dialysate than in the original DNA. (d) In measure with the alterations in the composition of the dialyzable fragments, the constitution of the dialysis residue is changed during digestion (Curve II in Figs. 3 to 6). In the case of T-DNA the preponderance of adenine over guanine and of thymine over cytosine shows a steady increase which becomes particularly steep when about 90 per cent of the DNA has been removed. The highest ratios thus obtained were adenine to guanine 2.2; thymine to cytosine 2.2; adenine to cytosine 4.4; purines to pyrimidines 2.3. This should be contrasted with the lowest values found for the dialysate, which were, in the same order, 1.1, 1.1, 1.0, and 0.8.

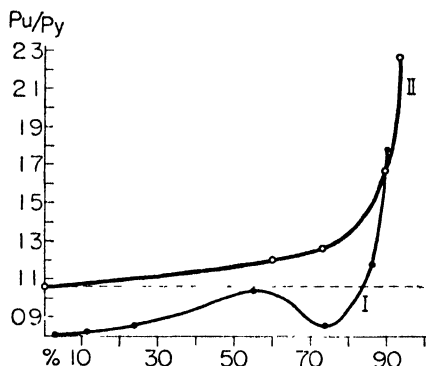


FIG. 6. Changes in the ratios of purines to pyrimidines in dialysate (Curve I) and dialysis residue (Curve II). See Fig. 3 for other explanations.

Interpretations—The purpose of this study was not so much the investigation of the mechanism of DNase action as an attempt to gain an insight into some of the principles that underlie the arrangement of nucleotides in the DNA molecule. Very little can, in fact, be said as yet about the action and specificity of the enzyme. Since no mononucleotides could be demonstrated, it would, for instance, be incorrect to state that the enzyme shows a preference for the splitting off of pyrimidine nucleotides, because the dialysate is relatively richer in pyrimidines. The polynucleotides, making up the readily dialyzable fraction, could very well consist of agglomerations of pyrimidine nucleotides flanked by purine nucleotides as end groups. Moreover, the possibility cannot be excluded that in the enzymatic degradation of DNA two effects are superimposed: an enzymatic cleavage of covalent linkages, followed by an automatic rupture of secondary valence bonds.

The findings on the course of disintegration of DNA presented here

could, at first sight, be interpreted as demonstrating the presence in the preparations of two differently composed types of DNA, one of which is distinguished by a higher degree of resistivity to enzymatic attack. This explanation cannot yet be dismissed entirely, but has found no support in the experiments with the ultracentrifuge reported above. A more plausible interpretation of the results is that T-DNA, which is a complicated high polymer, is composed of tracts of polynucleotides, differing in the proportions, and therefore in the sequence, of their components and in their susceptibility to enzymatic attack. The more resistant portion of the molecule, here termed the core, is characterized by a relatively much higher content in adenylic acid. It appears to be, in some manner, protected from the action of the enzyme, until other parts of the molecule, perhaps those bordering it, are split off. After the dialysis of 75 to 80 per cent of DNA fragments, the composition of the dialysate changes rather abruptly (Figs. 3 to 6), signifying the beginning of the diffusion of the core or of its breakdown products.

It appears not unlikely that DNase first breaks the DNA into several non-dialyzable fragments of which some contain the core, *i.e.* clusters or stretches of nucleotides, distinguished by greater resistance to enzymatic attack. As the digestion proceeds, the fragments devoid of core are completely degraded to easily dialyzable products; those that contain it are split gradually, with the core protected until exposed. The experiments described here were carried out with two different T-DNA preparations; they have shown a remarkable consistency in the sequence in which the enzymatic destruction took place. It will be of great interest to extend these observations to DNA from other species, especially to substances such as the DNA of avian tubercle bacilli (3), in which guanine and cytosine are the major constituents.

SUMMARY

Crystalline desoxyribonuclease, acting on the desoxyribose nucleic acid of calf thymus, yields dialyzable fragments and a dialysis residue (core). The distribution of individual purines and pyrimidines in both fractions was determined by a modification of previously described methods and was found to vary characteristically with the extent of digestion, thus suggesting a complex pattern of the sequence of individual nucleotides in the original nucleic acid. The core, as compared with the original nucleic acid, was characterized by greatly increased ratios of adenine to guanine, thymine to cytosine, purines to pyrimidines, and by greater resistance to enzymatic attack. A few experiments with the desoxyribonucleic acid of yeast are included. The bearing of these studies on the problem of the structure of desoxypentose nucleic acids is discussed.

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THE BIOLOGICAL FORMATION OF SARCOSINE*

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Methylglycine was first prepared in 1847 by Liebig (1) as a product of the degradation of creatine by a concentrated solution of barium hydroxide. Liebig named the new compound sarcosine. Despite its early preparation, it appears that the occurrence of sarcosine in higher animals has never been demonstrated. Indeed the only reference we have been able to find on the occurrence of sarcosine in nature is the report of Kossel and Edlbacher (2) in 1915 on the isolation of sarcosine from the radial ceca of the starfish, *Astropecten aurantiacus*. In the present communication evidence is presented that sarcosine is a metabolite in the rat.

The problem of sarcosine formation in this species was approached by applying the non-isotopic carrier technique to the intact animal. Sarcosinuria was produced by feeding rats a diet containing 5 per cent sarcosine. After several days, 0.1 per cent radiobetaine hydrochloride was added to the diet of one animal and 0.1 per cent radiomethionine was added to the diet of a second animal in the expectation that these radiomethyl compounds would contribute to the formation of any sarcosine that might be produced by the rat. The urine excreted during the next 24 hours was collected and sarcosine was isolated as the β -naphthalene-sulfonyl derivative. The β -naphthalenesulfonylsarcosine obtained in each of the experiments was recrystallized several times and examined for the presence of C^{14} . Both preparations were found to be radioactive.¹

Evidence that the radioactivity was not due to a contaminant was provided by converting the β -naphthalenesulfonylsarcosine to sarcosine hydrochloride without a loss in specific activity. Furthermore, when the sarcosine hydrochloride was converted to creatine hydrate, the specific activity remained unchanged.² Additional evidence that the C^{14} was a

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¹ Similar results were obtained when 1 gm. of non-isotopic sarcosine and 4.3 mg. of radiobetaine hydrochloride were injected intraperitoneally during the course of 24 hours.

² It was shown in control experiments that the radioactivity of the isolated sarcosine could not be attributed to contamination with radioactive creatine or radioactive creatinine.

part of the sarcosine molecule was obtained by converting the creatine hydrate to creatinine hydrochloride and thence to creatinine potassium picrate without a loss in specific activity. The specific activities of these compounds are given in Table I.

Samples of the radiosarcosine hydrochloride were degraded with silver oxide and the methylamine was isolated as the chloroplatinate. The radioactivity of the methyl carbon accounted for all of the C^{14} present in the radiosarcosine isolated in both the betaine and methionine experiments (Table I). These results indicate that sarcosine is formed by the rat and that the methyl groups of betaine and methionine are sources of the methyl carbon of sarcosine.

TABLE I

Specific Activity of Sarcosine Isolated As β -Naphthalenesulfonyl Derivative from Urine of Rats Fed Betaine or Methionine Labeled with C^{14} in Methyl Group

Sarcosine derivative	Compound fed	
	Radiobetaine	Radiomethionine
	c.p.m. per mm	c.p.m. per mm
β -Naphthalenesulfonylsarcosine.....	3.2×10^4	5.8×10^3
Sarcosine hydrochloride.....	3.2×10^4	5.3×10^3
Creatine hydrate	3.4×10^4	5.2×10^3
Creatinine chloride	3.2×10^4	5.4×10^3
“ potassium picrate	3.2×10^4	5.8×10^3
Methylamine chloroplatinate*.....	3.5×10^4	6.3×10^3

* Prepared from the radiosarcosine hydrochloride. Since the chloroplatinate contains 2 moles of methylamine per mole, its specific activity is expressed as counts per minute per 0.5 mm.

It is of interest to estimate the percentage of radiomethyl groups converted to sarcosine under the conditions employed. In both the betaine and methionine experiments, approximately 15 per cent of the ingested non-isotopic sarcosine was recovered from the urine. If it is assumed that the radiosarcosine recovered from the urine represented 15 per cent of the radiosarcosine synthesized, it may be calculated that 5.3 per cent of the methyl carbon of the ingested radiobetaine and 2.8 per cent of the methyl carbon of the ingested radiomethionine were converted to sarcosine in the body in 24 hours. Since betaine contains 3 times as many methyl groups as methionine and has a lower molecular weight than methionine, it may be calculated from these percentages, and from the food consumption, that approximately 8 times as much radiosarcosine was formed from the radiobetaine hydrochloride as from the radiomethionine. In this connection it should be noted that Dubnoff (3) has shown that homocysteine is methyl-

ated in the presence of betaine to yield methionine, and Muntz (4) has shown that dimethylglycine is a product of the reaction. If all, or most, of the radiosarcosine formed in our betaine experiment arose from the direct conversion of betaine to sarcosine by demethylation (with dimethylglycine as an intermediate), then approximately 16 per cent of the dietary betaine was converted to sarcosine. However, the conversion of significant quantities of betaine methyl carbon to sarcosine methyl carbon through other reactions cannot be excluded.

The experiments reported in this paper are of particular interest in view of parallel investigations (5) carried out in this laboratory on the metabolism of sarcosine. Direct proof of the conclusion of Handler, Bernheim, and Klein (6) that formaldehyde is produced in the oxidation of sarcosine by homogenized liver was provided by isolating formaldehyde from liver homogenates incubated with sarcosine. The origin of the formaldehyde from the methyl group was shown conclusively in experiments with radiosarcosine. Radioformaldehyde was also isolated from surviving liver slices incubated with radiosarcosine, and a trace of radioformaldehyde was found in the urine of a rat injected with radiosarcosine. Similarly, formic acid was shown to be a product of the oxidation of the methyl group of sarcosine by liver homogenates, liver slices, and the whole animal.

Still another single carbon compound has been identified as a product of sarcosine oxidation by a biological preparation. In 1944, Ratner, Nocito, and Green (7) reported that sarcosine was converted to methylamine and glyoxylic acid by a highly purified preparation of glycine oxidase obtained from the acetone-insoluble fraction of minced hog kidney. The methylamine was isolated and identified as the picrolonate.

EXPERIMENTAL

Metabolism Experiments—Two male rats of the Rockland Farms strain, weighing approximately 125 gm. each, were placed on a purified diet containing 5 per cent of non-isotopic sarcosine.* After maintaining a constant weight for 2 days the rats began to gain at a rate of approximately 3 gm. a day. No toxic symptoms were observed during the course of the experiments.

On the 6th day, 0.1 per cent of betaine hydrochloride labeled with C^{14} in the methyl groups (9) was added to the diet of one animal and 0.1 per cent of L-methionine labeled with C^{14} in the methyl group (10) was added to the diet of the second animal. The urine excreted during the next 24 hours was collected in 1 ml. of 1 N hydrochloric acid. During the collection

* The percentage composition of the basal diet was as follows: sucrose 50.4, casein 20, Covo 19, corn oil 1, L-cystine 0.4, betaine 0.2, sarcosine 5, Salts 4 (8), and vitamins (8).

period the rat fed radiobetaine ate 11.6 gm. of diet and the one given radiomethionine ate 8.4 gm. of diet.

Isolation of β -Naphthalenesulfonylsarcosine—Sarcosine was isolated from urine as the β -naphthalenesulfonyl derivative by a modification of the procedure described by Friedmann (11). The urine was adjusted to pH 8 to 9 with sodium hydroxide and filtered. 750 mg. of β -naphthalenesulfonylchloride in 25 ml. of ethyl ether were added and the mixture was stirred vigorously for 3 hours. The pH was maintained at 8 to 9 during the course of the reaction by the addition of sodium hydroxide when necessary. At the end of 3 hours, the ether phase was removed and the aqueous phase was extracted twice with 35 ml. portions of ether.

The aqueous phase was filtered, acidified to Congo red with hydrochloric acid, and placed in the refrigerator overnight. The β -naphthalenesulfonylsarcosine was removed by filtration and recrystallized twice from alkaline solution by the slow addition of hydrochloric acid, and twice from hot 22 per cent ethanol. The capillary melting point of the β -naphthalenesulfonylsarcosine isolated in both experiments was 171–172°, corrected. 280 mg. of the derivative were isolated from the urine of the rat which was fed radiobetaine and 178 mg. from the urine of the rat given radiomethionine. These quantities are equivalent to 15.2 per cent and 13.4 per cent, respectively, of the carrier sarcosine ingested during the 24 hour collection period.

52.3 mg. of the β -naphthalenesulfonylsarcosine isolated in the radiobetaine experiment were transferred to a filter paper disk and counted by the procedures described in an earlier publication (12). The crystals gave 1008 c.p.m. above background, or 6038 c.p.m. when corrected for self-absorption. The activity of the total amount of β -naphthalenesulfonylsarcosine isolated from the urine was 5.36 times this figure, or 3.24×10^4 c.p.m., corrected. This was equivalent to the activity of 93 γ of the ingested radiobetaine hydrochloride. If it is assumed as a first approximation that the radiosarcosine recovered from the urine represented 15.2 per cent of the quantity formed by the rat (15.2 per cent of the ingested carrier sarcosine was excreted), then 612 γ of the 11.6 mg. of ingested radiobetaine hydrochloride, or 5.3 per cent, were converted to sarcosine during the 24 hours of the experiment. On the basis of methyl groups, 1 molecule of betaine is equivalent to 3 molecules of sarcosine. In these experiments all of the C^{14} of the isolated sarcosine was located in the methyl group, as is shown below. Therefore, approximately 1.1 mg. of methyl-labeled sarcosine were formed from the radiobetaine in 24 hours. Similarly, 2.8 mg. of non-isotopic sarcosine were formed from the non-isotopic betaine consumed in the diet during the same period.

In the radiomethionine experiment, 47.4 mg. of the β -naphthalene-

sulfonylsarcosine isolated from the urine gave 182 c.p.m. above background, or 992 c.p.m. when corrected for self-absorption. The corrected value for the total amount of sarcosine derivative isolated was 3.75 times this figure, or 3.72×10^3 c.p.m. This was equivalent to the activity of 31 γ of the ingested radiomethionine. On the basis of the 13.4 per cent of carrier sarcosine isolated from the urine, it was calculated that 231 γ of the 8.4 mg. of ingested radiomethionine, or 2.8 per cent, were converted to sarcosine. Thus 0.14 mg. of methyl-labeled sarcosine was formed from the ingested radiomethionine in 24 hours. If the metabolism of the methionine of the dietary casein followed the metabolism of the radiomethionine, then an additional 1 mg. of sarcosine was synthesized from *exogenous* non-isotopic methionine during this same period, since casein contains approximately 3.5 per cent methionine (13).

The number of counts per minute per mm of the β -naphthalenesulfonylsarcosine isolated in the betaine and methionine experiments is given in Table I.

Sarcosine Hydrochloride—100 to 300 mg. of β -naphthalenesulfonylsarcosine were suspended in 2 ml. of concentrated hydrochloric acid and heated in a sealed tube at 100° for 22 hours (14). The β -naphthalenesulfonic acid crystals were removed by filtration and washed with a small amount of ice-cold hydrochloric acid. The hydrochloric acid was removed from the filtrate at the water pump and the residue was dissolved in warm absolute alcohol. The sarcosine hydrochloride was crystallized four times from alcoholic solution by the slow addition of ether. It was then decolorized with Darco and finally recrystallized from hot glacial acetic acid.

Prior to hydrolysis, the radioactive β -naphthalenesulfonylsarcosine isolated in the radiobetaine experiment was diluted 3-fold with non-isotopic β -naphthalenesulfonylsarcosine. The material isolated in the radiomethionine experiment was converted directly to sarcosine hydrochloride, recrystallized, and diluted 3-fold with non-isotopic sarcosine hydrochloride. In both experiments the capillary melting point of the radiosarcosine hydrochloride was 170°, corrected. For convenience of comparison, the specific activity of these and subsequent preparations has been calculated on the basis of the undiluted material (Table I).

Creatine and Creatinine—25 mg. of radiosarcosine hydrochloride were treated with cyanamide under the conditions described in an earlier publication (15). The crystals of creatine hydrate were centrifuged, and washed several times with a few drops of ice-cold water. The creatine hydrate was recrystallized from water by the addition of alcohol and its radioactivity measured in the usual manner.

The creatine hydrate was converted to creatinine hydrochloride by refluxing in 2 ml. of 1 N hydrochloric acid for 3 hours. The creatinine

hydrochloride was recrystallized from water and acetone and its specific activity⁴ determined.

Creatinine potassium picrate was prepared from the creatinine hydrochloride, and the potassium picrate was recrystallized once from 0.2 per cent picric acid half neutralized with potassium hydroxide, and once from water. The creatinine potassium picrate obtained in both experiments was pure according to analysis by the Jaffe reaction. The specific activities of the compounds described above are given in Table I.

Methylamine Chloroplatinate—20 mg. samples of the radiosarcosine hydrochloride prepared in each of the two experiments were degraded with silver oxide according to the method of Herbst and Clarke (16). The methylamine formed was aerated into 1 N hydrochloric acid. The acid was removed *in vacuo*, the residue was dissolved in 4 to 5 ml. of absolute alcohol, and methylamine chloroplatinate was precipitated by the dropwise addition of a saturated alcoholic solution of platinum tetrachloride. The methylamine chloroplatinate, following three recrystallizations from water and alcohol, melted on the micro block at 223–224°, corrected. The platinum content of both preparations agreed with the calculated value.

In each experiment the radioactivity of the methylamine chloroplatinate accounted for the C¹⁴ content of the isolated sarcosine. Since the chloroplatinate contains two methyl groups per mole, the specific activity was calculated as counts per minute per 0.5 mm (Table I).

Control Experiments—In order to justify the conversion of the isolated radiosarcosine to radiocreatinine as a test of the purity of the former compound, it was necessary to show that any radiocreatinine or radiocreatine that might have been excreted in the urine of the experimental animals was not carried along as a contaminant in the isolation of sarcosine. The following experiments were therefore carried out. 100 mg. of non-isotopic sarcosine and either 10.2 mg. of radiocreatinine⁴ (8.5×10^4 c.p.m., corrected) or 10 mg. of radiocreatine⁴ (7.3×10^4 c.p.m., corrected) were dissolved in 1 ml. of 1 N hydrochloric acid in the collection flask of a metabolism apparatus. The urine excreted by a rat in the next 24 hours was collected and sarcosine was isolated as the β -naphthalenesulfonyl derivative. In both instances the recrystallized derivative, when counted in pads of "infinite" thickness, gave less than 1 c.p.m. above the background.

In similar experiments either 1 mg. of radiobetaine hydrochloride or 1 mg. of radiomethionine was placed in the collection flask together with 100 mg. of non-isotopic sarcosine. The β -naphthalenesulfonylsarcosine isolated in the radiobetaine experiment gave 22 c.p.m. above the background for a

⁴ Diluted samples of the radiocreatinine and radiocreatine described in a previous publication (15).

pad of "infinite" thickness. This activity was negligible compared with the activity of the β -naphthalenesulfonylsarcosine isolated from the urine of the rat fed radiobetaine. Moreover, when the material isolated in the control experiment was converted to sarcosine hydrochloride, the counts dropped to less than 1 c.p.m. above the background.

In the radiomethionine experiment, the β -naphthalenesulfonylsarcosine, when counted in a pad of "infinite" thickness, gave 460 c.p.m. above background. However, when this material was converted to sarcosine hydrochloride, the counts dropped to 86 c.p.m. for a 34 mg. sample, and the creatine hydrate (16 mg.) derived from the sarcosine gave less than 1 c.p.m. above background. Thus, any C^{14} present in the urine of the experimental animals in the form of radiomethionine was eliminated in the conversion of the isolated sarcosine to creatine.

The authors wish to express their appreciation to Professor Vincent du Vigneaud for his advice and counsel during the course of this investigation. We wish to thank Mrs. Josephine T. Marshall for the platinum determinations.

SUMMARY

Rats were fed a diet containing non-isotopic sarcosine together with radiobetaine or radiomethionine labeled with C^{14} in the methyl group. Sarcosine was isolated from the urine as the β -naphthalenesulfonyl derivative and found to contain an appreciable degree of radioactivity.

Evidence that the C^{14} was incorporated in the sarcosine molecule was provided by converting the β -naphthalenesulfonylsarcosine through the following series of compounds without a loss in specific activity: sarcosine hydrochloride, creatine hydrate, creatinine chloride, and creatinine potassium picrate. The specific activity of the methyl group of the isolated sarcosine accounted for all of the C^{14} present in the molecule.

These results indicate that sarcosine is a metabolite in the rat and that "biologically labile" methyl groups are sources of the carbon of the methyl group of sarcosine.

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THE INACTIVATION OF BIOTIN BY HYDROCHLORIC ACID*

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Evidence has been presented for the existence in natural materials of substances with "biotin-like" activity, which, however, differ from biotin in their relative growth-promoting potencies for various microorganisms (1-6). In order to obtain further information on the occurrence of such substances, a study of the biotin activity of extracts prepared from natural products by a variety of procedures was undertaken. Observations on the stability of the biotin activity of natural substances and of *d*-biotin to hydrochloric acid are recorded in the present paper.

EXPERIMENTAL

In the initial experiments, 1 gm. of peptone,¹ solvamin,² or grass juice powder was autoclaved at 120° with 30 ml. of 2 N hydrochloric acid for 1½ hours and filtered. The filtrates were neutralized with sodium hydroxide to the pH suitable for the method employed and diluted to an appropriate volume, and the biotin activity was determined by microbiological assay with the following microorganisms: *Lactobacillus arabinosus* (2),³ *Lactobacillus casei* (7),⁴ *Streptococcus faecalis* R (8),⁵ and *Saccharomyces cerevisiae* 139 (9).⁶ It may be seen from Table I that peptone and solvamin exhibited comparable activities for all of the microorganisms. The grass juice powder, however, although possessing considerable activity for both *L. arabinosus* and *S. cerevisiae*, was inactive for *L. casei* and *S. faecalis*. In contrast, extracts prepared from the grass juice powder by autoclaving with 2 N sulfuric acid in a similar fashion possessed activity for *L. casei* and *S. faecalis*, as well as for *L. arabinosus* and *S. cerevisiae*. With all four microorganisms, *d*-biotin, added to neutralize hydrochloric and sulfuric acid extracts of grass juice powder, was quantitatively recovered. Therefore, the possibility of growth-inhibitory or growth-stimulatory substances in such extracts seems unlikely.

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¹ Difco Bacto-peptone.

² A dried extract from corn processed with *Clostridium acetobutylicum*.

³ L-Asparagine (0.01 per cent) was added to the basal medium.

⁴ American Type Culture Collection No. 7489.

⁵ Folic acid (2 γ per liter of medium) was added and the peptone omitted.

⁶ The cultures were grown in 30 ml. Erlenmeyer flasks at 30°.

The comparative effects of 2 N hydrochloric and sulfuric acids upon pure *d*-biotin were next determined. Solutions of *d*-biotin (30 μ gm. per ml.) in these acids were autoclaved at 120° for 1½ hours, then cooled, neutralized, and assayed. The hydrochloric acid treatment led to an almost complete loss of the growth-promoting activity for *L. casei* and *S. faeca-*

TABLE I
Biotin Content of Natural Materials

Material	Biotin content determined with			
	<i>L. arabinosus</i>	<i>L. casei</i>	<i>S. faecalis</i>	<i>S. cerevisiae</i>
	γ per gm.	γ per gm.	γ per gm.	γ per gm.
Solvamin*	0.57	0.76	0.61	0.88
Peptone*	0.22	0.23	0.22	0.24
Grass juice powder*	0.48	<0.03	<0.03	0.78
" " " †	0.82	0.50	0.51	1.31

* Autoclaved with 2 N hydrochloric acid.

† Autoclaved with 2 N sulfuric acid.

TABLE II
Effect of Hydrochloric Acid and Sulfuric Acid upon Growth-Promoting Activity of d-Biotin and dl-Oxybiotin

Compound	Acid employed	Activity* determined with			
		<i>L. arabinosus</i>	<i>L. casei</i>	<i>S. faecalis</i>	<i>S. cerevisiae</i>
<i>d</i> -Biotin	HCl†	50 (40-74)	1.2 (1.0-2.0)	1.3 (1.0-1.8)	44 (30-70)
	H ₂ SO ₄ †	100	95	105	103
<i>dl</i> -Oxybiotin	HCl†	105	102	102	97
	H ₂ SO ₄ †	100	100	101	95

* Expressed as the per cent of original *d*-biotin or *dl*-oxybiotin activity remaining after autoclaving with acid.

† The figures represent mean values calculated from the results of fourteen separate experiments. The range of values is given in parentheses.

‡ The figures represent mean values calculated from the results of five separate experiments. All observed values were within 10 per cent of the mean.

lis and approximately 50 per cent loss for *L. arabinosus* and *S. cerevisiae* (Table II). The sulfuric acid treatment was without effect. Thus the pure *d*-biotin and the biotin-active substance or substances of grass juice powder were affected similarly by the two acids.

Similar experiments (Table II) showed that solutions of *dl*-oxybiotin (60 μ gm. per ml.) (10) were stable to hydrochloric acid under these conditions. Since biotin differs from oxybiotin only in possessing a sulfur

atom in place of an oxygen atom in the ring, it appeared likely that this sulfur atom, which is readily oxidized (11-13), might be involved in its inactivation. This possibility was supported by the observation that the microbiological spectrum of *d*-biotin sulfoxide⁷ was similar to that of hydrochloric-inactivated *d*-biotin. Thus, *d*-biotin sulfoxide was as active on a molar basis as *d*-biotin for *L. arabinosus* and *S. cerevisiae* and was inactive for *L. casei* and *S. faecalis*.

These results are in agreement with those reported by du Vigneaud (13) and Melville *et al.* (12) and suggested that *d*-biotin sulfoxide was one of the products of the reaction of *d*-biotin with hydrochloric acid. The decrease in the activity of *d*-biotin for *L. arabinosus* and *S. cerevisiae* following treatment with hydrochloric acid may be due to its partial conversion into biotin sulfone or *l*-biotin sulfoxide. Biotin sulfone has no growth-promoting activity for any of the microorganisms under the conditions of our experiment (13, 14), while *l*-biotin sulfoxide (one of the diastereoisomers formed in the oxidation of *d*-biotin) was found by Melville *et al.* (12) to be inactive for *S. cerevisiae* and approximately 5 per cent as active as *d*-biotin for *L. arabinosus*.

With all four microorganisms, *d*-biotin added to neutralized solutions of hydrochloric acid-treated biotin was quantitatively recovered. As with hydrochloric acid-treated grass juice powder, experiments of this type render unlikely the possibility that the lack of activity for *L. casei* and *S. faecalis* is due to the presence of inhibitory substances. No loss of biotin activity for *L. casei* and *S. faecalis* was noted when the hydrochloric acid was neutralized *prior* to the addition of *d*-biotin and the resulting solution autoclaved.

Hydrochloric acid, prepared by dissolving hydrogen chloride (liberated by the action of sulfuric acid upon hydrochloric acid) in water which had been triply distilled through an all-glass apparatus, also inactivated *d*-biotin. Similar inactivation was noted with hydrochloric acid prepared by double distillation of a constant boiling mixture of c.p. hydrochloric acid through an all-glass apparatus.

A number of natural materials⁸ were autoclaved with both hydrochloric and sulfuric acids, as previously described, and the biotin activities of the resulting extracts determined with *L. arabinosus* and *S. faecalis*. For each organism, comparable values were obtained with both acids. Thus, the grass juice powder was the only natural material investigated which showed

⁷ Prepared by saponifying the methyl ester with normal sodium hydroxide for 2 hours at room temperature.

⁸ Difco yeast and malt extracts; rice polish concentrate; skim milk powder; dried whole egg; whole liver substance, liver fraction L, and liver powder 1:20 (The Wilson Laboratories); rat liver, kidney, and muscle; chick leg muscle.

the inactivating effect of hydrochloric acid. In an attempt to explain this unique behavior of grass juice powder, it was postulated that this material lacks a protective "mechanism" against hydrochloric acid in-

TABLE III

*Recovery of d-Biotin Autoclaved with Hydrochloric Acid in Presence of Peptone or Grass Juice Powder**

Peptone (1 gm.)	Peptone (1 gm.) + 0.20 γ biotin	Per cent recovery of added biotin	Grass juice powder (1 gm.)	Grass juice powder (1 gm.) + 0.50 γ biotin	Per cent recovery of added biotin
<i>L. arabinosus</i>					
0.22 (0.20-0.25)	0.40 (0.36-0.43)	91 (80-105)	0.48 (0.44-0.50)	0.67 (0.58-0.74)	38 (28-48)
<i>S. faecalis</i>					
0.22 (0.20-0.24)	0.42 (0.38-0.44)	103 (85-115)	<0.03	<0.03	

* The mean values (expressed as micrograms of *d*-biotin) of five separate experiments are presented. The range of values is given in parentheses. 1 gm. of substance alone or with the indicated amounts of *d*-biotin was autoclaved with 30 ml. of 2 N hydrochloric acid for 1½ hours.

TABLE IV

*Biotin Activity of Grass Juice Powder Autoclaved with Hydrochloric Acid in Presence of Peptone**

Substance†	Biotin content determined with	
	<i>L. arabinosus</i>	<i>S. faecalis</i>
	γ	γ
Peptone (0.5 gm.)	0.11 (0.10-0.13)	0.11 (0.11-0.12)
Grass juice powder (0.5 gm.)	0.24 (0.22-0.25)	<0.015
Peptone (0.5 gm.) + grass juice powder (0.5 gm.)	0.48 (0.42-0.51)	0.38 (0.30-0.42)

* The mean values of four separate experiments are presented. The range of values is given in parentheses.

† 1 gm. of the individual substance or of the mixture was autoclaved with 30 ml. of 2 N hydrochloric acid for 1½ hours.

activation which is present in other natural products. Data supporting this hypothesis are presented in Table III, where it can be seen that *d*-biotin autoclaved with hydrochloric acid in the presence of peptone could be

quantitatively recovered for *L. arabinosus* and *S. faecalis*. This was in direct contrast to the inability of grass juice powder to prevent the inactivation of added *d*-biotin by hydrochloric acid (Table III). Further confirmation was given by the observation that the presence of peptone during autoclaving with hydrochloric acid caused a marked increase in the biotin activity of grass juice powder (Table IV). Thus, peptone is protective both for *d*-biotin and for the substance or substances in grass juice powder possessing biotin activity.

DISCUSSION

The data presented in this paper demonstrate that the growth-promoting activity of *d*-biotin for certain microorganisms is markedly impaired by treatment with hydrochloric acid. It seems likely that *d*-biotin is converted by hydrochloric acid into a mixture of the corresponding *d*- and *l*-sulfoxides and biotin sulfone. The possibility exists that trace impurities (e.g., chlorine) in the hydrochloric acid may be responsible for this effect. It should be emphasized that the present studies have been conducted with extremely dilute biotin concentrations (30 m μ gm. per ml.), which are frequently encountered in the extraction of natural materials for biotin assay. Brown and du Vigneaud observed that the yeast growth activity of biotin was destroyed when more concentrated biotin solutions were autoclaved with 20 per cent hydrochloric acid for protracted periods of time (15).

The microbiological spectrum of biotin treated with a dilute solution of hydrogen peroxide (16) and rancid fat (17) is similar to that of the hydrochloric acid-treated biotin observed in the present studies. In all cases, the partial oxidation of the sulfur atom of biotin is probably involved.

The existence of biotin complexes in nature is well known, and the recent isolation of biocytin from yeast extract is further evidence that the biotin activity of natural materials may be due to a compound or compounds differing structurally from biotin (3). In view of the undetermined nature of the compounds contributing to the biotin activity of grass juice powder, any attempts to explain the action of hydrochloric acid upon this material would be premature. Whatever the mechanism may be, the possible deleterious effects of hydrochloric acid should be considered when this acid is employed in the extraction of biotin from natural materials.

SUMMARY

1. Autoclaving *d*-biotin with hydrochloric acid results in almost complete loss of activity for *Lactobacillus casei* and *Streptococcus faecalis* and approximately 50 per cent destruction for *Lactobacillus arabinosus* and *Sac-*

charomyces cerevisiae. A consideration of the microbiological spectra of *d*- and *l*-biotin sulfoxides and biotin sulfone suggests that *d*-biotin is converted into a mixture of these compounds through the action of hydrochloric acid.

2. The growth-promoting activities of *d*-biotin and of grass juice powder for various microorganisms are affected similarly by hydrochloric acid. This inactivating effect of hydrochloric acid is not shown by a variety of other natural materials.

3. Autoclaving with sulfuric acid has no adverse effect upon the growth-promoting activity of biotin or grass juice powder.

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MICROBIOLOGICAL ACTIVITY OF THE GUANIDO ANALOGUES OF BIOTIN AND OXYBIOTIN*

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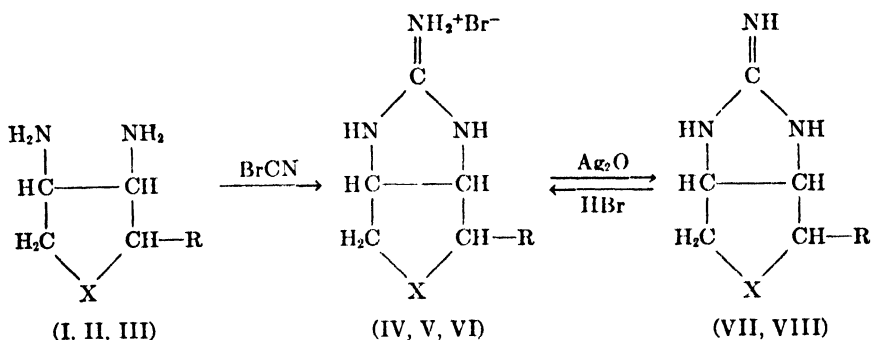
Recent studies have established a high degree of biological specificity of the biotin molecule. Structural alterations of this vitamin have usually resulted in compounds which were either inactive as growth promoters or which were antagonistic to biotin (1). One exception is the biologically active compound, oxybiotin, in which the sulfur atom of biotin is replaced by oxygen (2). Little information is available regarding the effects of modifications of the urea portion of the biotin molecule. Opening of the urea ring to give *cis*-3,4-diamino-2-tetrahydrothiophenevaleric acid resulted in a marked loss of biological activity (3).

The fact that ammonia is required for the demonstration of the fermentation-stimulating ability of biotin (4) and oxybiotin (5) in *Saccharomyces cerevisiae* suggested the possibility that these compounds might function in ammonia transfer through the intermediate formation of their guanido analogues. Consequently, we have synthesized the guanido analogues of both *d*-biotin and *dl*-oxybiotin and have determined their microbiological activities. These experiments also furnish information regarding the specific requirements for the cyclic urea portion. The preparation of the desired cyclic guanido compounds was first investigated with the model compound hexahydro-2-imino-4-methyl-1-furo-(3,4)-imidazole hydrobromide (IV).

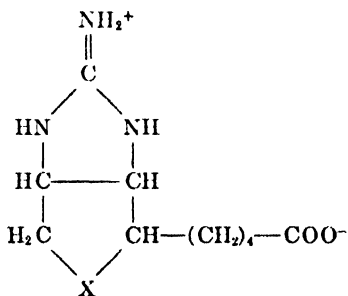
Starting materials for the present investigation were the sulfates of the diamino compounds (I, II, and III), which were obtained from *dl*-hexahydro-2-oxo-4-methyl-1-furo-(3,4)-imidazole, *dl*-oxybiotin, and *d*-biotin, respectively, by drastic hydrolysis with barium hydroxide (6, 7, 2). The free diamino compounds were liberated from their sulfates by the addition of theoretical amounts of barium hydroxide and treated with cyanogen bromide in aqueous solution (8). This reaction in the case of *cis*-3,4-diamino-2-methyltetrahydrofuran and *cis*-3,4-diamino-2-tetrahydrofuran-valeric acid led to the formation of the hydrobromides of the desired guanido derivatives (IV and V). In the case of the diaminocarboxylic

* Supported in part by grants from Ciba Pharmaceutical Products, Inc., and the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

acid (III) derived from *d*-biotin, a mixture of the free guanido analogue (VIII) and of its hydrobromide (VI) was obtained. The failure of the guanido derivatives to liberate nitrogen under the conditions of the Van Slyke amino nitrogen determination demonstrates that ring closure had occurred. The free guanido analogue of oxybiotin (VII) was obtained from its hydrobromide (V) by treatment with silver oxide. Compounds VII and VIII most likely possess zwitter ionic structures of the types depicted.



(I, IV), X = O and R = —CH₃; (II, V and VII), X = O and R = —(CH₂)₄—COOH; (III, VI and VIII), X = S and R = —(CH₂)₄—COOH.



X = O or S.

When tested for ability to replace biotin in the nutrition of *S. cerevisiae*, *Lactobacillus arabinosus*, and *Lactobacillus casei*, compounds VII and VIII were found to be practically devoid of activity (0.002 to 0.009 per cent as active as *d*-biotin). The slight activities observed were probably due to traces of oxybiotin or biotin, respectively. Autoclaving with dilute sulfuric acid had no effect upon the microbiological activity, demonstrating that the compounds were not converted into either oxybiotin or biotin by this treatment. It is, therefore, apparent that substitution of the urea portion

in biotin or oxybiotin by a guanido moiety completely destroys the growth-promoting activity.

EXPERIMENTAL

Preparation of Compounds¹

Hexahydro-2-imino-4-methyl-1-furo-(3,4)-imidazole Hydrobromide (IV)—To a solution of 214 mg. of *cis*-3,4-diamino-2-methyltetrahydrofuran sulfate (I) in 5 ml. of water, 20 ml. of 0.1 N barium hydroxide were added and the resulting barium sulfate was removed by filtration. To the clear filtrate 106 mg. of cyanogen bromide were added and the mixture was shaken until a clear solution resulted. The solution was kept at room temperature for 2 hours and was then concentrated to a small volume *in vacuo*. The resulting crystals, following recrystallization from absolute ethanol, melted at 186–188°. The yield was 176 mg. A sample for analysis was dried *in vacuo* over P₂O₅ at 100° for 5 hours.

Analysis—C₆H₁₁ON₃HBr. Calculated. C 32.46, H 5.44, N 18.92, Br 36.00
Found. " 32.33 " 5.46 " 19.34 " 36.20

Hexahydro-2-imino-1-furo-(3,4)-imidazole-4-valeric Acid Hydrobromide (V)—To a solution of 300 mg. of *cis*-3,4-diamino-2-tetrahydrofuran-valeric acid sulfate (II) (2) in 2 ml. of water, 0.5 N barium hydroxide solution (4 ml.) was added and the resulting barium sulfate removed by filtration. Cyanogen bromide (157 mg.) was added to the clear filtrate and the mixture shaken until all of the cyanogen bromide had dissolved. The solution was kept at room temperature for 2 hours and was then heated on a steam bath for an additional hour. Evaporation of the solution *in vacuo* yielded crystals which were washed with acetone and recrystallized from a mixture of acetone and ethyl acetate. Yield, 243 mg.; m.p., 174–175°.

Analysis—C₁₀H₁₇O₃N₃HBr. Calculated. C 38.97, H 5.88, N 13.63, Br 25.93
Found. " 38.99 " 6.02 " 13.55 " 26.18

Hexahydro-2-imino-1-furo-(3,4)-imidazole-4-valeric Acid (VII)—To a solution of 166 mg. of (V) in 5 ml. of water was added an excess of freshly prepared silver oxide and the mixture shaken for a few minutes at room temperature. The precipitate of silver oxide and silver bromide was removed by filtration and hydrogen sulfide was passed into the solution in order to remove the excess of silver ions. The resulting silver sulfide was removed by filtration and the clear filtrate concentrated to dryness *in*

¹ The melting points were determined with short stem Anschütz thermometers and are uncorrected.

vacuo. Recrystallization of the residue from dilute ethanol gave 80 mg. of needles melting with decomposition at 295–300°.

Analysis— $C_{10}H_{17}O_2N_2$. Calculated. C 52.86, H 7.54, N 18.48
Found. " 52.32 " 7.50 " 18.26

Hexahydro-2-imino-1-thieno-(3,4)-imidazole-4-valeric Acid (VIII)—To a hot solution of 316 mg. of *cis*-3,4-diamino-2-tetrahydrothiophenevaleric acid sulfate (III) (7) in 2 ml. of water, 0.5 N barium hydroxide (4 ml.) was added and the resulting precipitate of barium sulfate removed by filtration. The precipitate was washed with two 1 ml. portions of hot water and 175 mg. of cyanogen bromide were added to the cooled combined filtrates and washings. The mixture was shaken until all of the cyanogen bromide had dissolved. After 10 minutes at room temperature, needle-like crystals began to form in the solution. The mixture was kept at room temperature for 2 hours and was then placed in a refrigerator for an additional 12 hours in order to complete the crystallization. The crystals were collected and air-dried. Yield, 90 mg. Recrystallization from water and air-drying at room temperature gave a hydrate of (VIII) which decomposed above 260°. The substance gave no color with ninhydrin and was free of halogen. Drying of the hydrate at 100° over P_2O_5 for 5 hours resulted in a weight loss of 17.3 per cent and afforded the anhydrous substance as a highly hygroscopic powder.

Analysis— $C_{10}H_{17}O_2N_2S$. Calculated. C 49.36, H 7.04, N 17.27
Found. " 49.37 " 7.16 " 17.30

Hexahydro-2-imino-1-thieno-(3,4)-imidazole-4-valeric Acid Hydrobromide (VI)—The mother liquors from the preparation of (VIII) above were concentrated to a small volume *in vacuo* and cooled in an ice bath. The resulting crystals were collected and recrystallized from water to which a drop of 48 per cent hydrobromic acid had been added. Drying over P_2O_5 *in vacuo* afforded 80 mg. of (VI) melting at 235–236°. This compound also resulted from the treatment of (VIII) with hydrobromic acid.

Analysis—
 $C_{10}H_{17}O_2N_2SHBr$. Calculated. C 37.04, H 5.59, N 12.96, S 9.89, Br 24.64
Found. " 37.18, " 5.57, " 12.58, " 10.08, " 25.12

Microbiological Procedures

The ability of compounds VII and VIII to replace biotin as a growth factor was determined for the following microorganisms: *S. cerevisiae* (9), *L. arabinosus* (10), and *L. casei* (11). Experiments were also conducted in which the two analogues were sterilized by filtration through a glass filter and added under sterile conditions to the medium. The results obtained with this procedure were identical with those observed when the

samples were autoclaved with the medium. Autoclaving of the analogues for 2 hours at 120° in 4.5 N sulfuric acid had no effect on their activity for *L. arabinosus*.

SUMMARY

The guanido analogues of *d*-biotin and *dl*-oxybiotin have been synthesized by treatment of the respective *cis*-diaminocarboxylic acids with cyanogen bromide. These compounds were found to be practically devoid of microbiological activity for *Saccharomyces cerevisiae*, *Lactobacillus arabinosus*, and *Lactobacillus casei*, thus emphasizing the biological specificity of the cyclic urea portion of biotin and oxybiotin.

The authors wish to express their gratitude to Dr. Karl Folkers of Merck and Company, Inc., for a generous gift of *d*-biotin. The technical assistance of Miss M. Elizabeth Lybarger is gratefully acknowledged.

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FREE γ -AMINOBUTYRIC ACID IN BRAIN*

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(Received for publication, March 27, 1950)

The presence of unidentified amino compounds in tissue extracts has been revealed by means of two-dimensional paper chromatography. Rat brain appeared to contain an amino compound which occupies a position in the chromatogram corresponding to that of γ -aminobutyric acid. No other organ examined contained this material.

Although γ -aminobutyric acid has been reported to be present in potato extracts (1) and only in trace amounts in blood and urine (2), no report has been found in the literature concerning its occurrence in tissues of higher animals. While this paper was being written, Reed (3) reported the occurrence of this compound in yeast, and, independently from us, Roberts (4) reported on its occurrence in brain. Gale, in his excellent paper (5) on bacterial decarboxylases, pointed out that glutamic acid is converted quantitatively to γ -aminobutyric acid by the action of certain strains of bacteria. The possibility that a similar mechanism may be operating in brain was considered. This paper deals with the isolation, identification, and estimation of free γ -aminobutyric acid in the brains of human, rat, guinea pig, rabbit, pigeon, and beef.

EXPERIMENTAL

Preparation of Beef Brain Extract—About 3 kilos of fresh beef brain were homogenized in a Waring blender with about 3 liters of 95 per cent alcohol. The thick homogenate was placed on a boiling water bath until the proteins had coagulated. The coagulated material was filtered through filter paper pulp in a Büchner funnel. The clear filtrate was evaporated to about 300 ml. To remove lipide material from the extract, it was treated with about 500 ml. of chloroform and was centrifuged until the emulsion was broken. The aqueous layer was collected and filtered. A clear, dark amber-colored extract was obtained which was evaporated to dryness in the water bath. The syrupy residue was suspended in 300

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ml. of absolute ethanol and was boiled for 5 minutes. The suspended solids were filtered off while hot, and the filtrate was saved for further work. The residue was extracted for 8 hours with absolute ethanol in a Soxhlet extractor. This extract was combined with the first alcoholic extract and was then evaporated to a small volume. The concentrated extract was placed in a desiccator containing absolute ethanol. After 48 hours, a material crystallized out which contained no amino nitrogen. It was filtered off and discarded. This operation was repeated three more times. The final concentrated extract was tested by paper chromatography, and was found to contain large amounts of an unknown substance in addition to alanine, and small amounts of glutamic acid, leucine, and glycine.

Isolation of Unknown—The isolation of a pure fraction containing the unknown was accomplished by chromatography on starch. A column 42×225 mm. was prepared with 300 gm. of potato starch previously suspended in the solvent. The solvent used was 2:1:1 *n*-butanol-acetic acid-water. A 2 gm. aliquot of the extract was dissolved in about 5 ml. of the solvent and applied to the column. The effluent was collected in 1 ml. fractions in a Technicon automatic fraction collector. The unknown was located by means of paper chromatography. Four more columns were prepared under identical conditions, and the remainder of the extract was fractionated. The contents of all of the tubes containing only the unknown were pooled, and the solvent was evaporated in a water bath. A thick syrup remained which was dissolved in 10 ml. of water, decolorized with Darco, and evaporated under a vacuum. The residue was dissolved in 0.2 ml. of water, and then absolute ethanol was added. Crystals of the unknown appeared which were collected and dried over silica gel. The material thus obtained was highly hygroscopic and gave no clear cut melting points. It decomposed at about 200° .

Identification of Unknown—The isolated material was tested chromatographically. It gave single, homogeneous spots with all solvents tried. The silver salt was prepared, and the nitrogen and the silver contents were determined. Found, N 7.13 per cent, Ag 51.2 per cent; calculated, N 6.65 per cent, Ag 51.4 per cent. The unknown substance must be an aminobutyric acid, as indicated by the silver salt analysis. The unknown was chromatographed on filter paper, together with synthetic γ -aminobutyric acid, β -aminoisobutyric acid, α -aminobutyric acid, and α -aminoisobutyric acid. The results obtained are shown in Fig. 1. Resolution was obtained in all cases except in the case of the synthetic γ -aminobutyric acid, which shows a homogeneous, single spot. β -Aminobutyric acid does not give a color with ninhydrin up to 20 γ (3). Further evidence was obtained by chromatography on paper in different solvents. The R_f values were identical for both the isolated material and the syn-

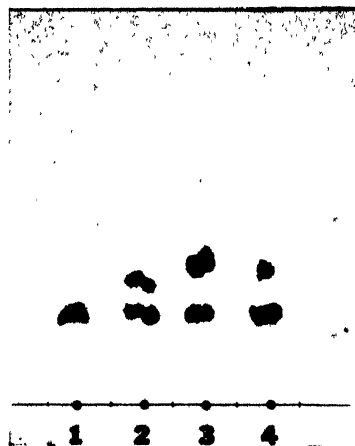


FIG. 1. Paper chromatogram of mixtures of the isolated material with (1) synthetic γ -aminobutyric acid, (2) β -aminoisobutyric acid, (3) α -amino-*n*-butyric acid, and (4) α -aminoisobutyric acid. About 10 γ of each acid were used and chromatographed with 2,4-lutidine-water.

TABLE I

Approximate Concentration of γ -Aminobutyric Acid in Brain of Various Species

The results are in micrograms per gm. of fresh tissue.

Type of brain	Total amino nitrogen	γ -Aminobutyric acid nitrogen
Human. Pons	388	60
“ Temporal lobe	400	75
“ Frontal “	415	77
“ Parietal “	400	70
“ Occipital “	444	62
“ Caudate nucleus	479	103
“ Cerebellum	403	86
“ Medulla	330	62
“ Gray matter	348	65
“ White “	338	32
Guinea pig	292	100
Rat	523	86
Pigeon	470	110
Rabbit	257	38
Beef	413	69

thetic γ -aminobutyric acid. The R_F values found were 0.05 in butanol, 0.26 in 2,4-lutidine, 0.74 in phenol, and 0.70 in butanol-acetic acid. These data show definitely that the substance isolated from brain is γ -aminobutyric acid.

Estimation of γ -Aminobutyric Acid in Brain—The approximate concentration of γ -aminobutyric acid in brain was determined by the method described by Landua and Awapara (6). In all instances, the specimens were analyzed within a few minutes after removal from the animal. In the case of human brain, analysis was made within 6 hours after death. The total amino nitrogen was calculated from the yield of color with ninhydrin. The figures reported serve as a reference for approximating the percentage of γ -aminobutyric acid in relation to total free amino nitrogen. They do not represent absolute values.

RESULTS AND DISCUSSION

The approximate concentration of γ -aminobutyric acid in brain of various animal species is reported in Table I. The human brain was dissected into its component parts for analysis.

The values found suggest that γ -aminobutyric acid is an important fraction of the free amino nitrogen of brain. Any evaluation of its importance in brain metabolism would be only speculation at this time. No experimental data are available to suggest the metabolic fate of this substance in brain. However, there are sufficient data on hand to indicate that γ -aminobutyric acid arises in brain from glutamic acid. When rat or rabbit brain homogenates at pH 7 were incubated with glutamic acid, a small but definite portion of the glutamic acid which disappeared could be accounted for by a parallel increase in the γ -aminobutyric acid fraction. When extracts of acetone-dried powder were used, different results were obtained. Anaerobically, in 2 hours, 60 to 70 per cent of the glutamic acid which disappeared may be accounted for in the form of γ -aminobutyric acid. Since the measurement of the latter was carried out chromatographically, other amino acids could be detected simultaneously. Aspartic acid increased at the end of the 2 hour period, but the increase was too small to measure with accuracy. The product of the reaction with glutamic acid was in the same position on the chromatogram as γ -aminobutyric acid and was stable to cupric carbonate (7), indicating that it was not an α -aminocarboxylic acid. Also, it gave homogeneous, single spots when chromatographed together with synthetic γ -aminobutyric acid.

The presence of free γ -aminobutyric acid in brain and its possible formation from glutamic acid suggest that there is a new type of enzyme in brain capable of decarboxylating glutamic acid.

SUMMARY

1. The presence of free γ -aminobutyric acid has been established chromatographically. Proof of its identity was provided by isolation of the

compound and by the silver salt. Analysis of the latter showed close correlation with the calculated values.

2. The probable origin of γ -aminobutyric acid was investigated. The data showed that glutamic acid is converted into γ -aminobutyric acid by decarboxylation.

The authors wish to acknowledge Dr. L. Carroll King of Northwestern University for the analysis of the silver salt and Dr. Lester J. Reed of The University of Texas for the sample of β -aminoisobutyric acid.

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THE DISTRIBUTION OF COBALT IN POLYCYTHEMIC RATS*

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(Received for publication, June 17, 1950)

The mechanism of the hematopoietic action of cobalt in the rat is still unknown. The usual analytical methods, with the exception of spectroscopy, are not sufficiently sensitive to determine the concentration of the administered cobalt in the blood cells; however, with the use of radioactive cobalt these determinations can be satisfactorily carried out. Stare and Elvehjem (1), who were limited to the determination of the amount of cobalt in the entire body of the rat, found that 0.04 to 0.05 mg. of cobalt in the entire body was sufficient to produce a polycythemia. In two rats fed a diet containing 1 per cent cobalt sulfate for 7 weeks, Josland (2) found that per 100 gm. of wet tissue the spleens contained 1.79 and 0.62 mg. of cobalt, that the kidneys contained 2.12 and 1.7 mg., and the livers 3.75 and 4.36 mg.

This study was undertaken to determine the distribution in the rat of the injected cobalt during the development of the polycythemia in the tissues known to have a hematopoietic function and in the kidneys to see whether some clue to the mechanism of the hematopoietic action of cobalt could thereby be found.

Methods

Blood counts were made with standard pipettes and a standard hemocytometer. The tissue distribution of cobalt was determined by a modification of the method of Weymouth, Wasserman, Berlin, and Rosenthal (3) suggested by R. W. Dunn.¹ The animal was perfused with saline through the inferior vena cava. The liver, spleen, and left kidney and lung were removed. A 1.0 cc. sample of blood was obtained before starting the perfusion. A small fraction of the liver and the entire spleen, kidney and lung were placed in tared quartz centrifuge cones. The femur was removed and the bone cut across just above the condyles. With a dry No. 20 gage needle, the marrow was aspirated and transferred to a quartz centrifuge cone. To the wet tissues in the quartz centrifuge cones was added 0.1 cc. of a saturated solution of calcium nitrate per gm. of tissue. The tissues were dried, placed in a cold muffle furnace, and ashed,

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† Formerly Post Doctorate Research Fellow of the National Cancer Institute.

¹ Personal communication to the author.

by a method suggested by Dunn,¹ at 650° for 2 hours. The ash was dissolved in hydrochloric acid and then transferred to a centrifuge cone and 1.0 cc. of a 10 per cent solution of hydroxylamine added. The solution was made basic with ammonium hydroxide, at which point a dense white precipitate formed. The solution was centrifuged, and the supernatant decanted and saved. The precipitate was then dissolved in hydrochloric acid, hydroxylamine and ammonium hydroxide were added, and it was again centrifuged. The process was repeated for a total of four extractions. The combined supernatants were then added to an electroplating cell, which contained 5 mg. of carrier cobalt as cobaltous sulfate and 15 cc. of a 2 per cent solution of ammonium sulfate in concentrated am-

TABLE I

Red Blood Cell Count and Hemoglobin of Rats Given Intraperitoneally 0.125 Mg. of Cobalt Daily

Time	Red blood cell count	Hemoglobin
<i>wks.</i>	<i>million per c.mm.</i>	<i>gm. per 100 cc.</i>
Initial	9.8	16.0
1	10.8	16.4
2	11.8	17.0
3	12.5	17.5
4	13.5	18.0
5	13.8	19.0
6	14.4	20.0
7	15.2	20.0
8	15.5	20.5
9	14.7	19.5
10	14.9	22.0
11	15.9	22.3
12	15.3	22.3
13.5	16.5	23.5
15	17.0	22.0

monium hydroxide. This was electroplated at 200 ma. for 2 hours in an apparatus designed by Dunn.² The plates were removed from the electroplating cell and dried. The Co⁶⁰ was assayed by counting with a thin mica end window Geiger-Müller tube.

Experimental Procedure

A series of twenty-nine rats was given intraperitoneally 1.0 cc. of a solution containing 0.125 mg. of cobalt as cobaltous sulfate, 0.2 mg. of iron as

¹ Dunn, R. W., Donner Laboratory of Medical Physics, University of California, unpublished observations.

ferric chloride, and a tracer amount of Co^{60} , and buffered with sodium citrate to pH 5.4. The tissue distribution of the injected cobalt was determined at 3, 6, 9, 24, and 48 hours following a single intraperitoneal injection, and after daily injections (six per week) for 2, 4, 7, 18, 29, 35, 39, 55, 69, 83, 92, and 105 days. The latter group of animals was perfused 3 hours following the last injection.

Results

Table I shows the increase in red blood cells and hemoglobin. The distribution of the injected cobalt in the spleen, red blood cells, and bone

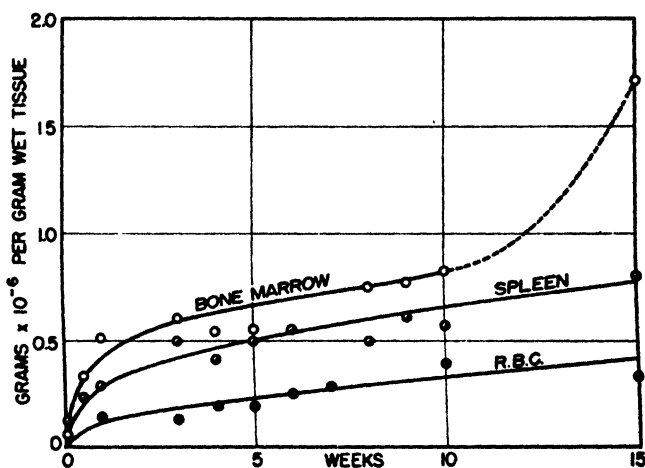


FIG. 1. The distribution of injected cobalt in the bone marrow, spleen, and red blood cells.

marrow is presented in Fig. 1, and that in the liver, kidney, and lung in Table II. Table III shows the distribution at 3, 6, 9, 24, and 48 hours following a single intraperitoneal injection of cobalt.

DISCUSSION

The previous investigators (1, 2) of the distribution of cobalt in the rat were limited to the determination of the amount present in either the whole body (1) or in the liver, kidney, pancreas, and spleen. As a consequence of the small amount of cobalt present in the blood, especially in the red blood cells and bone marrow, they were probably unable to determine the cobalt content of these tissues.

Table III shows that following a single intraperitoneal injection cobalt is rapidly taken up by the tissues and, although the majority leaves the

tissues rapidly, a small amount is retained for longer periods of time. The bone marrow, spleen, and red blood cells show an increase during the course of cobalt injections; the liver, kidney, and lung rapidly reach a maximum value, which, however, can differ within rather wide limits from animal to animal.

Since the cobalt which is taken up by the tissues is largely excreted rapidly, and since there is, during the course of cobalt administration, no

TABLE II

Distribution of Cobalt Injected in Tissues in Gm. $\times 10^{-6}$ per Gm. of Wet Tissue

Sacrificed at, days	2		4		7		18		29		35	
Animal No.	7	8	9	10	11	14	30	31	26	27	28	29
Liver.	1.2	1.1	1.3	1.8	1.6	1.2	2.6	2.3	2.4	2.6	1.8	0.9
Kidney.	0.9	0.9	1.2	1.4	2.0	1.4	1.8	1.7	1.7	1.9	1.4	1.7
Lung	0.2	0.2	0.2	0.3	0.3	0.3	0.4	0.2	0.2	0.3	0.1	0.3
Sacrificed at, days.	39		55		58		69		83	92	105	
Animal No. . . .	32	33	21	22	17	18	19	20	35	37	24	25
Liver	2.0	1.8	2.1	1.7	3.6	2.9	2.2	3.5	2.4	1.2	1.2	2.1
Kidney.	1.3	0.9	1.9	1.6	1.7	1.8	1.8	1.7	1.5	1.2	1.3	1.6
Lung	0.2	0.2	0.4	0.4	0.3	0.3	0.4	0.3	0.2	0.3	0.2	0.3

TABLE III

Distribution of Injected Cobalt in Tissues in Gm. $\times 10^{-6}$ per Gm. of Wet Tissue Following Single Intraperitoneal Injection of 0.125 Mg. of Cobalt

Time of sacrifice, hrs.	3	6	9	24	48
Kidney.		0.9	0.9	0.6	0.4
Liver.	1.2	0.9	0.5	0.4	0.4
Spleen.	0.2	0.2	0.2	0.1	0.1
Lung.	0.2	0.2	0.3	0.2	0.1
Whole blood.	0.2	0.2	0.3	0.1	0.04

great increase in the concentration of cobalt, except in the bone marrow, the cobalt concentration as determined in these experiments is almost entirely derived from the last dose. Any variation from animal to animal in the uptake by a particular organ, and, more significantly, any variation in the rate of excretion from a given organ, can lead to large variations in the concentration of cobalt as determined in these experiments.

The increase in cobalt concentration in the bone marrow is possibly a result of replacement of fat by hematopoietic tissue which Saviani and

Baccari (4) demonstrated chemically by a decrease in the lipide content of the bone marrow of rabbits with cobalt-produced polycythemia.

SUMMARY

1. The distribution of injected cobalt in the liver, spleen, kidney, lung, blood, and bone marrow of polycythemic rats is presented.

2. The cobalt rapidly reaches a saturation level in the liver, kidney, and lungs, and, with a trend toward a slow increase in the red blood cells, in spleen and bone marrow on prolonged administration.

The author wishes to thank Dr. John H. Lawrence and Dr. John W. Gofman for their assistance in the preparation of this report and in carrying out this study.

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THE EFFECTS OF FOLIC ACID AND AMINOPTERIN UPON ENZYME SYSTEMS IN VITRO*

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The influence of folic acid upon xanthine or xanthopterin oxidase activity measured *in vitro* has been studied by various workers (1-4). In 1948 Kalckar and Klenow (1) observed that xanthopterin oxidase from cream is strongly inhibited by approximately equimolar amounts of folic acid. They also showed later that this inhibitory effect is given to a much less extent by highly purified folic acid (2). It was observed from data on oxygen uptake (3) that it was possible to obtain inhibition of guanine metabolism in rat liver homogenates and xanthine oxidation by purified xanthine oxidase from cream even with highly purified folic acid if the level of inhibitor was increased high enough. It is possible, however, that trace amounts of photolytic products of folic acid, present in the vitamin preparation even after purification, were capable of inhibiting the enzyme systems studied, although almost duplicate results were obtained whether highly purified or ordinary commercial folic acid was used. Lowry *et al.* (4) subsequently demonstrated that the primary photolytic product of folic acid (2-amino-4-hydroxy-6-formylpteridine) is a remarkably powerful inhibitor of xanthine oxidase from cream.

Some interesting effects of dietary folic acid upon various animal enzyme systems have been observed recently. Keith *et al.* (5) reported that dietary folic acid inhibits xanthine oxidase activity of chick liver. In our laboratory these results were confirmed and extended to various other enzyme systems (6). It was observed that dietary folic acid strongly inhibits chick liver D-amino acid oxidase activity, although no effect of dietary folic acid upon endogenous respiration of liver or on liver catalase activity could be demonstrated. Dinning *et al.* (7) found that if aminopterin, a powerful folic acid antagonist, was fed to monkeys liver and kidney choline oxidase activity was greatly depressed. They were unable to demonstrate any consistent effect of folic acid or aminopterin upon choline oxidase activity when those substances were added

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to the enzyme system *in vitro*. Recently we have reported (8) that choline oxidase activity of chick embryo liver is depressed by folic acid fed to the mother hen. A depressant effect was also observed upon xanthine oxidase activity in chick embryo liver.

In the present paper we wish to report results concerning the effects of folic acid and aminopterin on various enzyme systems in rat liver, in which the vitamin or antagonist was added *in vitro* to liver homogenates from healthy animals. Most of the enzyme systems discussed above were studied in order to observe whether a direct relationship between the effects of (dietary) folic acid and aminopterin *in vitro* and *in vivo* could be established.

Experimental and Results

General—All liver homogenates employed in these experiments were prepared from livers of adult, male rats of the Holtzman strain maintained on a good stock ration. Homogenization was effected by rapidly extirpating the liver from decapitated animals, chilling the liver in cracked ice, and homogenizing it in the appropriate buffer with a Potter-Elvehjem homogenizer.¹

Solutions of folic acid and aminopterin were prepared by adding dilute ammonium hydroxide to dry samples of the substances until they were just in solution and then diluting to volume with water.

Effect of Folic Acid and Aminopterin on Endogenous Respiration of Liver—The rat livers were homogenized in 6 volumes of 0.039 M sodium and potassium phosphate buffer (pH 7.3) (9). As rapidly as possible, 2 ml. portions of the resulting homogenate were pipetted into prepared Warburg flasks chilled in cracked ice. The side arms of the flasks contained the following amounts of folic acid or aminopterin in duplicate: 0, 41.5, 83, and 126 γ . Water was added to make each side arm contain 0.5 ml. final volume. After a 10 minute equilibration period at 30° the substrates and water were tipped into the homogenate, and readings of oxygen uptake were taken at 15 minute intervals for 30 minutes.

The results of a typical experiment of this sort are presented in Fig. 1. Here the 15 minute oxygen uptake is plotted against levels of folic acid or aminopterin added to the homogenate. From the curves it can be seen that both folic acid and aminopterin inhibit endogenous respiration of liver to approximately the same extent. Moreover, the inhibition approaches a maximum at the lowest level of inhibitor added (41.5 γ) and remains constant for higher concentrations of inhibitor. The maximum

¹ We wish to thank the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for the supplies of folic acid and aminopterin used in this investigation.

inhibition is slightly less than 50 per cent of the normal endogenous respiration. Since a considerable portion of endogenous respiration of liver is undoubtedly due to oxidation of endogenous purine substrates and since folic acid (or its primary photolytic product) is known to inhibit both guanine and xanthine catabolism *in vitro* (3), it is probable that the inhibition of endogenous respiration reported in Fig. 1 is due primarily to inhibition of the enzyme systems catabolizing endogenous purines.

During the early endogenous respiration studied in the foregoing experiment, it is probable that the oxidation of endogenous purines is maximum and that, if the liver homogenate is incubated until most of the endogenous purines are removed, the inhibitory effects of folic acid and aminopterin should be greatly decreased. In testing this concept, liver homogenates were prepared as described and incubated without addition

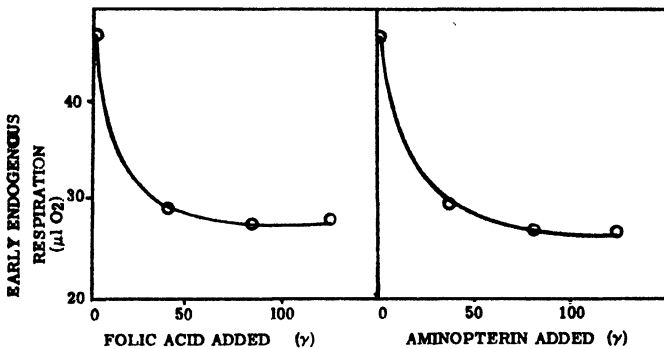


FIG. 1. The effect of various levels of folic acid and aminopterin upon early endogenous respiration after homogenization of rat liver.

of folic acid or aminopterin for 90 minutes in Warburg flasks at 30°. The folic acid or aminopterin was then added from the side arms. Oxygen uptake readings were taken at the end of 30 minutes. In Fig. 2 are presented the results of a typical experiment of this sort. From these curves it can be seen that folic acid and aminopterin no longer inhibit the endogenous respiration of the liver.

From the results of Figs. 1 and 2 it appears then that the inhibition of endogenous respiration by folic acid and aminopterin might be due to a competition with certain endogenous substrates for the enzyme. When those endogenous substrates have been catabolized by incubating the homogenate alone, folic acid and aminopterin no longer inhibit the endogenous respiration. Since folic acid is known to inhibit enzymatic oxidation of some of the purines, it is probable that the endogenous substrates in question are purines.

Effect on Liver Xanthine Oxidase Activity—In the next series of experiments a comparison of the effects of folic acid and aminopterin on liver xanthine oxidase activity was made. To the side arms of a series of Warburg flasks folic acid or aminopterin was added at the following levels in duplicate: 0, 16.7, 167, and 1670 γ . Each flask contained 2 ml. of 16.7 per cent rat liver homogenate and 0.2 ml. of 10 per cent KOH in the center well. In addition to the folic acid or aminopterin in the side arms, 0.1 ml. of 0.0312 M xanthine was added to alternate flasks. Water was added to each side arm to bring the side arm volume to 0.5 ml. The flasks were incubated for 90 minutes at 30° to remove endogenous substrates, the side arm contents then tipped in, and oxygen uptake readings taken at 15 minute intervals for 30 minutes. The oxygen uptake due to xanthine oxidation was calculated by subtracting the endogenous activity from the corresponding flask containing added xanthine.

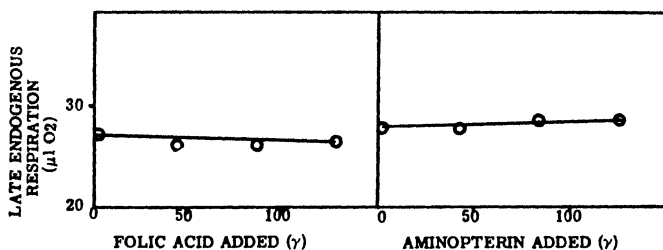


Fig. 2. The effect of folic acid and aminopterin upon endogenous respiration of rat liver after 90 minutes of incubation.

In Fig. 3 are presented the results of this type of experiment. From these graphs it can be observed that both folic acid and aminopterin inhibit rat liver xanthine oxidase activity to almost exactly the same degree. Increasing the levels of either inhibitor increases the degree of inhibition so that the highest level included inhibits the xanthine oxidase activity completely. It appears from the results that equimolar amounts of the vitamin and antagonist inhibit xanthine oxidase activity of rat liver to approximately the same degree.

Photolysis of Folic Acid and Aminopterin—Whether the inhibition of xanthine oxidase by both folic acid and aminopterin is due to photolytic products of the substances or to the intact compounds themselves is a question that has arisen since the reports of Lowry *et al.* (4, 10). We have investigated the photolysis of both folic acid and aminopterin by following the changes in fluorescence after irradiation of the compounds in solution. In these experiments 834 γ of the compounds in 5 ml. of 0.1 N acetic acid were irradiated with a 365 $m\mu$ mercury line with the

tubes containing the compounds placed 2 inches from the ultraviolet source. At 0, 15, 30, and 45 minute intervals, 0.1 ml. aliquots of each solution were removed and added to 9.9 ml. of 0.2 M borate buffer (pH 9.0). Fluorescence of the borated solutions was then measured with a Coleman model 12 fluorometer (thiochrome filters). From the results in Table I, it appears that both aminopterin and folic acid are photosensitive, giving rise to fluorescent compounds upon irradiation with ultraviolet light. It is suspected that the compound analogous to the photolytic product of folic acid (2-amino-4-hydroxy-6-formylpteridine) is

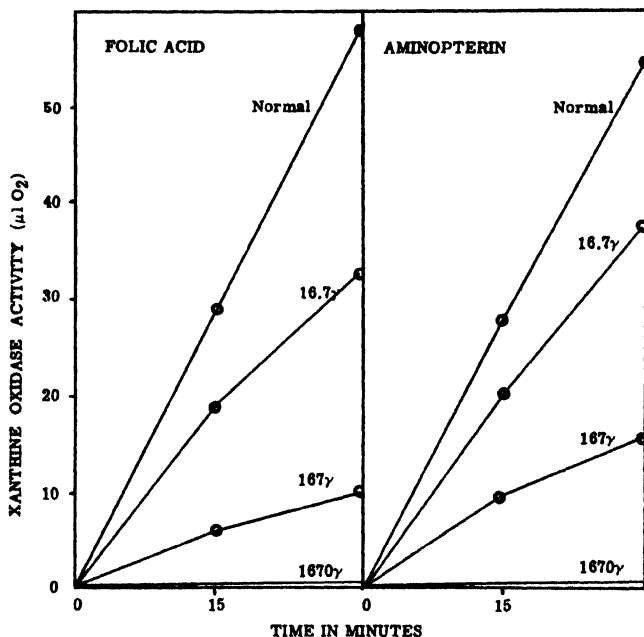


FIG. 3. The effect of various levels of folic acid and aminopterin upon rat liver xanthine oxidase activity.

formed from irradiation of aminopterin (2,4-diamino-6-formylpteridine). The rates of photolysis of both aminopterin and folic acid are approximately the same, although the reaction with aminopterin may occur slightly faster than with folic acid. Therefore, it appears that aminopterin gives rise to a photolytic derivative analogous to that obtained from folic acid. Whether this derivative is the 6-formylpteridine analogue of aminopterin is open to further investigation, however.

Effect on Choline Oxidase—We have recently reported (8) that the choline oxidase activity of liver from chick embryos is depressed by folic

acid fed to the mother hen. These results were very similar to the effect of folic acid in the diet of the mother hen upon embryonic liver xanthine oxidase. It appears then that in the case of xanthine oxidase dietary folic acid has the same general effect upon liver xanthine oxidase activity as does folic acid added *in vitro* to a normal liver xanthine oxidase system. In order to observe whether this result applies to other enzymes, the effect of folic acid and aminopterin added *in vitro* to a rat liver system oxidizing choline was studied. If the effect of folic acid added *in vitro* to the choline oxidase system is the same as the effect of maternal dietary folic acid upon chick embryo liver, folic acid should inhibit the rat liver enzyme system when added *in vitro*. Moreover, if the example of xanthine oxidase is followed, aminopterin added *in vitro* should also inhibit the choline oxidase system.

A series of Warburg flasks was prepared which contained 0.1 ml. of 10 per cent KOH in the center well and 1.0 ml. of 16.7 per cent rat liver

TABLE I
Photolysis of Folic Acid and Aminopterin upon Irradiation with Ultraviolet Light

Time of irradiation	Fluorescence (galvanometer divisions)	
	Folic acid	Aminopterin
<i>min.</i>		
0	0	0
15	5	9
30	12	18
45	17	20

homogenate in 0.039 M sodium and potassium phosphate buffer. To the side arms of alternate flasks was added 0.5 ml. of 0.5 per cent choline chloride or 0.5 ml. of water. Each of the following levels of folic acid or aminopterin was added to the side arms of two flasks: 0, 29, 71, and 96 γ . Extra water was added to all side arms to bring the final side arm volume to 1.0 ml. The flasks were incubated at 30° for 1 hour to remove endogenous substrates, and the side arm contents were then tipped in. Oxygen uptake readings were taken at 10 minute intervals for 30 minutes. Choline oxidase activity was calculated from the first 10 minute readings.

The effect of folic acid and aminopterin added *in vitro* to the choline oxidase system is presented in Table II. The results are given as microliters of oxygen uptake due to oxidation of choline for the first 10 minute period in the presence of various levels of folic acid or aminopterin. From these results it can be observed that neither folic acid nor aminopterin added to the choline oxidase system *in vitro* has any effect upon choline oxidase activity. It appears, therefore, that although both

dietary and folic acid *in vitro* bring about inhibition of liver xanthine oxidase activity, these results do not necessarily hold true for other enzyme systems. Since both dietary folic acid and aminopterin influence tissue choline oxidase activity markedly (7, 8), although they have no influence upon activity of this enzyme when added to it *in vitro*, the vitamin and antagonist, when fed, have their effect upon choline oxidase activity in some other way than by acting directly on the enzyme. As pointed out in an earlier report (8), dietary folic acid is probably involved in the regulation of the synthesis of certain enzyme proteins, *e.g.* choline oxidase, while it may act upon other enzymes, *e.g.* xanthine oxidase, directly.

TABLE II

Effect of Folic Acid and Aminopterin Added in Vitro upon Rat Liver Choline Oxidase Activity

	Level of vitamin or antagonist added	Choline oxidase activity
	γ	$\mu\text{l. O}_2$
Folic acid	0	53
	29	54
	71	55
	96	54
Aminopterin	0	54
	29	55
	71	54
	96	54

SUMMARY

1. Data have been presented which indicate that both folic acid and aminopterin, when added to enzyme systems *in vitro*, have very similar effects. The enzyme systems studied were endogenous respiration, xanthine oxidase, and choline oxidase of rat liver.

2. Both folic acid and aminopterin strongly inhibit rat liver xanthine oxidase to approximately the same degree. The substances have no effect upon choline oxidase activity of rat liver when added *in vitro*, although, if they are fed, they inhibit activity of this enzyme.

3. Our experimental evidence indicates that aminopterin is probably photolytically decomposed in the same manner as folic acid.

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γ -AMINOBUTYRIC ACID IN BRAIN: ITS FORMATION FROM GLUTAMIC ACID*

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Relatively large quantities of an unidentified ninhydrin-reactive material were found in numerous two-dimensional paper chromatograms of protein-free extracts of fresh mouse, rat, rabbit, guinea pig, human, and frog brains. At most, only traces of this material were found in a large number of extracts of many other normal and neoplastic tissues and in urine and blood. In the present experiments and in the following paper (1), the unknown compound in brain extract is conclusively identified as γ -aminobutyric acid. It is shown that this compound can be formed from glutamic acid by homogenates, washed residues, and acetone powders of brain. A preliminary report of some of these findings has been made (2).

EXPERIMENTAL

Chromatography.—One-dimensional and two-dimensional chromatograms were made by the descending method as outlined by Consden *et al.* (3) and extended by Dent (4, 5).

Separation of Unknown Material.—Mice were killed by dislocation of the cervical vertebrae, and the brains were removed immediately and frozen in dry ice. A pooled sample weighing 7.8 gm. was homogenized in 100 ml. of 75 per cent alcohol. After centrifugation, the extract was evaporated almost to dryness, taken up in 2.6 ml. of water, and the aqueous extract was centrifuged to remove insoluble material. This extract was then employed for chromatography.

In Fig. 1 is shown a tracing of a chromatogram obtained from an aliquot of extract corresponding to 75 mg. of fresh mouse brain. Intense spots were given by aspartic acid, glutamic acid, glycine, glutamine, taurine, alanine, and the unknown material. Cystine, serine, and β -alanine were present in smaller amounts, and traces of valine, threonine, and glutathione were noted. Two substances which disappeared on acid hydrolysis were located to the left of aspartic and glutamic acids on the chromatograms. Among the substances which have been studied chromatographically (5), the unknown material corresponded most closely in its behavior to histi-

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dine, methionine sulfoxide, and γ -aminobutyric acid. Its position on the chromatogram was especially favorable for separation from most of the other detectable constituents by one-dimensional chromatography with phenol, thus making possible a more adequate comparison of its properties with those of the aforementioned substances.

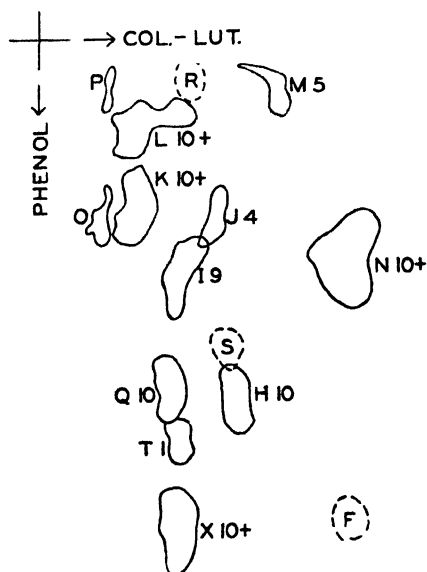


FIG. 1

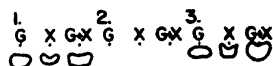


FIG. 2

FIG. 1. Tracing of a chromatogram of a peroxide-treated aliquot of brain extract corresponding to 75 mg. of fresh tissue. The letters identify the spots and the numbers represent visual estimates of relative intensities of color, a rating of 1 being taken as the color given by 1 γ of amino acid. The spots delineated by a broken line represent traces. M, cystine (cysteic acid); L, aspartic acid; K, glutamic acid; J, serine; I, glycine; N, taurine; Q, glutamine; H, alanine; T, β -alanine; S, threonine; F, valine; X, unknown substance; R, glutathione; P and O, unidentified materials which disappear on acid hydrolysis.

FIG. 2. Chromatography of unknown (X) and γ -aminobutyric acid (G) in (1) collidine-lutidine, (2) phenol, (3) butanol.

Ten spots of 10 μ l., each corresponding to 30 mg. of fresh tissue, were placed along the narrow edge of each of four sheets of Whatman No. 1 filter paper and run in water-saturated phenol for 24 hours. The two end strips of each sheet were cut out and sprayed with a 0.1 per cent solution of ninhydrin in butanol. Six bands appeared in all instances. The unknown material was located in the band furthest from the starting point. The most likely contaminant would be the small quantity of valine shown in Fig. 1. With the two end strips as reference guides, the strips of paper

containing the unknown material were cut out of the four chromatograms. All ninhydrin-reactive material was removed from the strips by allowing water to flow down them into beakers. No color was given when these strips were sprayed subsequently with ninhydrin after drying. The eluate was evaporated to dryness and taken up in 2 ml. of distilled water. Subsequent chromatography of aliquots of the eluate before and after hydrolysis with 6 N HCl showed the presence of a large quantity of the unknown substance, which was stable to hydrolysis, and traces of valine and a peptide material.

Identification of Unknown Compound As γ -Aminobutyric Acid—Microbiological assay for histidine¹ and a comparison of the chemical and chromatographic properties with those of histidine and methionine sulfoxide showed that the unknown was neither of these compounds.

A known sample of γ -aminobutyric acid² (15 γ) and the unknown compound (52 μ l.) were then chromatographed separately and in mixture in three different water-saturated solvent systems: *n*-butanol, phenol, and collidine-lutidine. The results are shown in Fig. 2. Although the R_f values (3) are widely different in the solvent systems employed, the main constituent of the unknown material and the γ -aminobutyric acid traveled at the same rate in each case and gave completely superimposable bands when mixed. It was therefore concluded that the unknown compound is γ -aminobutyric acid. This conclusion was confirmed by application of the isotope derivative method to the eluted material (1).

Quantitative Determination of γ -Aminobutyric Acid—Advantage was taken of the fact that this amino acid could be separated from all but traces of two of the ninhydrin-reactive materials found in brain by one-dimensional chromatography in phenol (see the previous discussion). By using smaller concentrations of brain extract the quantities of the interfering substances were reduced below a level at which positive reactions with ninhydrin were given. Under these conditions the color given by the γ -aminobutyric acid could be used for quantitative determination.

Spots corresponding to known amounts of γ -aminobutyric acid and accurately measured amounts of unknown samples were placed along the narrow edge of sheets of Whatman No. 1 filter paper and run in water-saturated phenol for 24 hours. After thorough removal of the phenol by directing a fan at the sheets for 18 to 24 hours, the sheets were sprayed on both sides with a 0.1 per cent solution of ninhydrin in butanol. Maximal color development took place in 24 to 36 hours at room temperature or in 30 minutes at 93°. The developed spots were cut out, care being taken

¹ The microbiological determinations were kindly performed by Dr. G. B. Ramasarma. The organism *Leuconostoc mesenteroides* P-60 was employed in the assay.

² γ -Aminobutyric acid was purchased from the Department of Chemistry, University of Illinois. It was recrystallized from alcohol.

to include the same total area of paper in all of the known and unknown samples in any set of determinations. Suitably chosen paper blanks were always included. The pieces of paper containing the spots were cut into small strips with minimal handling and were put into test-tubes. 5 ml. of water distilled in glass were then added and the tubes were shaken vigorously for a minute or two. Quantitative elutions of the color were achieved by this method. The paper fiber was then centrifuged and the color was read at 570 $m\mu$ in the Beckman spectrophotometer. The optical density was proportional to the concentration in the range studied (1 to 15 γ). The color was stable for at least 4 hours at room temperature when the tubes were kept out of strong light. Although the standard curves obtained at different times were in good agreement, a series of standards was run with each set of determinations in order to eliminate the influence of possible differences in paper and solvent on the development of the color. A similar procedure was employed in which the unknown and standards were run on two-dimensional chromatograms in phenol and collidine-lutidine. Good checks were obtained when brain extracts were analyzed by both the one-dimensional and two-dimensional methods. Three experimental samples of brain homogenates gave total values of 149, 202, and 278 γ of γ -aminobutyric acid by the one-dimensional procedure, while values of 156, 200, and 295 γ , respectively, were obtained for the same samples by two-dimensional chromatography. This indicates the adequacy of the one-dimensional method under the conditions of the experiment. Recoveries of γ -aminobutyric acid which had been added to brain homogenates ranged from 92 to 100 per cent. The presence of glutamic acid and aspartic acid in concentrations 200 times as great as those of the γ -aminobutyric acid did not affect the recovery.

The above methods have been applied successfully to the determination of several other amino acids in pure solutions and in protein hydrolysates.

γ -Aminobutyric Acid Present in Brain Chiefly in Free Form—The average value of a number of determinations of γ -aminobutyric acid in alcoholic extracts of mouse brain was 55 mg. per 100 gm. of fresh weight of tissue. Closely similar values were found in the supernatant solutions after heat coagulation of brain homogenates, in trichloroacetic acid extracts, and in acid hydrolysates of whole brain. It was also found that hydrolysis with 6 N HCl did not increase the content of γ -aminobutyric acid in the protein-free extracts. These results show that the γ -aminobutyric acid is present in brain chiefly in the free form.

Influence of Glutamic Acid on Content of γ -Aminobutyric Acid during Incubation of Brain Homogenates, Washed Residues, or Acetone Powders—Homogenates of freshly excised mouse brain containing 250 mg. of tissue per ml. were prepared in ice-cold 0.05 M phosphate buffer, pH 7.42. In

several experiments the homogenates were used directly. In others the homogenates were centrifuged in the cold at 3500 r.p.m., the supernatants discarded, and the residues washed with varying volumes of phosphate buffer one or more times. The residues were then suspended in a volume of buffer such that the final volume of the suspension was the same as that of the original homogenate. The acetone powders were prepared by suspending fresh brain in 100 to 500 volumes of acetone at -15° in a Waring blender, filtering rapidly with suction, resuspending in the same volume of fresh acetone, filtering, and finally drying *in vacuo* over P_2O_5 and paraffin at 4° . Suitable amounts of the powders were then suspended in phosphate buffer with the aid of a ground glass homogenizer prior to use.

Immediately after preparation of the suspensions, 2 ml. aliquots were pipetted into tubes containing 8.45 ml. of 95 per cent alcohol and 0.67 ml. of distilled water to serve as blanks. 2 ml. portions of the preparations were also added to 0.67 ml. of water or to 0.67 ml. of a solution containing a suitable concentration of glutamic acid, previously adjusted to pH 7.4. These latter mixtures were then incubated for 2 hours at 38° in an atmosphere of O_2 in a Dubnoff incubator. The reactions were stopped by the addition of 8.45 ml. of 95 per cent alcohol and the mixtures stirred thoroughly and centrifuged. The clear supernatants were poured off and aliquots were used for analysis. Whenever aliquots greater than 0.3 ml. were used, the two-dimensional chromatographic procedure was employed.

The data for representative experiments are summarized in Table I. In all of the preparations employed, the increase of γ -aminobutyric acid was accelerated by the presence of added glutamic acid. Placing the preparations in boiling water for 1 minute destroyed the activity. In several instances, there were increases in the content of γ -aminobutyric acid in the absence of added glutamic acid. This occurred to the largest extent in the crude homogenates. Even in the most extensively washed preparations there were still easily detectable amounts of those amino acids which were present in the fresh tissue in the free form to the largest extent. From this it would be expected that constituents of brain, other than those which react with ninhydrin, would also adhere to the washed residues or acetone powders. For this reason it was not possible to conclude from these experiments that glutamic acid was a precursor of γ -aminobutyric acid. The possibility was not ruled out that glutamic acid was indirectly stimulating the formation of γ -aminobutyric acid from some other substance. The following experiments were undertaken to determine whether or not the carbon of glutamic acid would be found in γ -aminobutyric acid after incubation with a preparation which was actively forming the latter compound.

Formation of γ -Aminobutyric Acid from Glutamic Acid by Brain Acetone

Powder—Glutamic acid which was uniformly labeled with C^{14} was kindly furnished to us by Dr. Konrad Bloch. This amino acid had been isolated from a hydrolysate of algae grown in $C^{14}O_2$. The glutamic acid had a specific activity of 27,900 c.p.m. per mg. when counted as the amino acid with a windowless proportional counter connected to a scaling unit. The samples were deposited from dilute aqueous solution on cups 10.7 sq. cm. in area. A sample containing 1 mg. of the labeled amino acid to which

TABLE I
Influence of Glutamic Acid on Formation of γ -Aminobutyric Acid in Brain Preparations

Incubation conditions described in the text. In the case of the homogenates and washed residues an amount of tissue equivalent to 500 mg. of original fresh weight of tissue was employed. 50 mg. of acetone powder were used.

Type of preparation	Experiment No.	Final molarity of added glutamic acid	Change in content of γ -aminobutyric acid per tube
Homogenate	1	0	γ 49
		0.1250	111
	2	0	50
		0.1250	100
Washed residue	1	0	-5
		0.0008	4
		0.0039	35
		0.0078	47
	2	0	22
		0.0078	69
		0.0313	85
		0.1250	179
		0.1250*	3
		0	8
Acetone powder	1	0.1250	369
		0.1250*	8
		0.0367	40
	2	0.1250	144
		0.1250	

* Heat-inactivated.

5 γ of γ -aminobutyric acid had been added was subjected to two-dimensional chromatography. At the same time a chromatogram was run containing 5 γ of γ -aminobutyric acid, and one containing a series of samples of known concentrations was run to serve as a standard curve. There were no ninhydrin-reactive constituents other than glutamic and γ -aminobutyric acids on the paper containing the radioactive glutamic acid. On elution and estimation of the quantity of γ -aminobutyric acid, 5 γ were

recovered. The eluate contained no radioactivity whatsoever. This shows that the sample of glutamic acid employed contained no γ -aminobutyric acid or other ninhydrin-reactive impurities, that no γ -aminobutyric acid was formed from the glutamic acid during the chromatographic procedures, and that no radioactive contaminants were present which migrated to the same position as the γ -aminobutyric acid on the chromatogram. The recovery from the paper containing γ -aminobutyric acid alone was 4.7 γ , a value within the range of experimental error.

An experiment was then performed in which 40 mg. of fresh acetone powder from mouse brain were incubated with 146,000 counts of glutamic acid (5.25 mg.) in a final volume of 1 ml. under the conditions previously described; 3.16 ml. of 95 per cent alcohol were then added. Samples of

TABLE II

Radioactivity Found in Eluates of Ninhydrin-Positive Areas from Two-Dimensional Chromatograms after Incubation of C^{14} -Labeled Glutamic Acid with Acetone Powder of Mouse Brain

146,000 counts of glutamic acid were added originally to the incubation mixture.

Amino acid	Total counts in sample
	<i>c.p.m.</i>
Glutamic acid.	127,800
γ -Aminobutyric acid	354
Glutamine..	20
Glycine .	0
Leucines	0
Valine ...	0
Alanine.....	0
Taurine	0

the clear supernatant (0.75 ml.) were subjected to two-dimensional chromatography. It was found that the total content of γ -aminobutyric acid after incubation exceeded by 40 γ that found in the zero time control.

The spots corresponding to glutamic acid and to all of the detectable constituents which were clearly separated from the large glutamic acid spot were eluted with suitable volumes of water, plated, and counted. In this manner it was possible to obtain results for glutamic acid, γ -aminobutyric acid, glutamine, glycine, the leucines, valine, alanine, and taurine. The counts are shown in Table II.

Of the constituents other than glutamic acid, by far the largest activity was found in the γ -aminobutyric acid. Glutamine was the only other constituent having a significant number of counts. The activity recovered in the glutamic acid spot (127,800 c.p.m.) was somewhat higher than would

have been expected if 1 mole of CO_2 had been liberated per mole of glutamic acid present (117,000 c.p.m.). The reaction with ninhydrin probably does not go to completion on paper when such large quantities of amino acid are present.

The above experiment shows conclusively that the carbon chain of glutamic acid can be converted to that of γ -aminobutyric acid. It is not possible at the present time to deal with the stoichiometry of the reaction because of the relatively low specific activity of the glutamic acid and the weak activity of the enzyme preparation employed. It appears highly probable, however, that the formation of γ -aminobutyric acid from glutamic acid in brain takes place by α decarboxylation.

Toxicity of γ -Aminobutyric Acid—Two mice, weighing approximately 20 gm., were given a single intraperitoneal injection of 100 mg. of γ -aminobutyric acid in 0.5 ml. of water, and two other mice were given 0.25 ml. of the same solution. There were no signs of toxicity and the mice appeared to be completely normal thereafter. This indicates that the acute toxicity of γ -aminobutyric acid is low.

DISCUSSION

An extensive survey of the free amino acids in many normal and malignant mouse tissues, a part of which has been reported (6, 7), revealed the presence of considerable quantities of a ninhydrin-reactive substance in extracts of brain which was present, at most, in traces in the other tissues examined. This substance was also detected in the brains of other species. The experiments reported in this paper and in the following one (1) established with certainty that this compound is γ -aminobutyric acid. Experiments with homogenates, washed residues, and acetone powders of mouse brain invariably showed increases in the content of γ -aminobutyric acid upon incubation with glutamic acid. With isotopically labeled glutamic acid, it was found that glutamic acid could serve as a precursor of γ -aminobutyric acid. Recent unpublished experiments in our laboratory with homogenates and sterile autolysates of liver, muscle, and tumors have shown increases of a substance which corresponds closely in its properties to γ -aminobutyric acid after incubation for varying periods of time. It may thus be that the formation of γ -aminobutyric is of wide occurrence in mammalian tissues. The uniquely high concentrations of this substance in brain may possibly be a result of a greater rate of formation than in other tissues, a slower rate of removal, or a combination of both. A study is being made of the metabolism of γ -aminobutyric acid.

γ -Aminobutyric acid has recently been reported to be present in bacteria (8), plants (9), human blood and urine (5), adult ox and embryo calf muscle (10), and yeast (11). In bacteria and plants, this compound can

arise as a result of the action of glutamic acid decarboxylase (12, 13). The presence of this enzyme has not yet been demonstrated in any of the other sources in which γ -aminobutyric acid has been reported. The experiments reported in this paper indicate that such an enzyme may be present in brain tissue.

SUMMARY

1. γ -Aminobutyric acid has been shown to be a constituent of brain in which it exists preponderantly in the free form.

2. Experiments with various crude enzyme preparations from mouse brain showed a net increase of γ -aminobutyric acid, which was accelerated by the addition of glutamic acid.

3. It was shown by the use of isotopically labeled glutamic acid that the latter amino acid can serve as a precursor for the γ -aminobutyric acid.

4. It is suggested that the formation of this compound in brain takes place by the α decarboxylation of glutamic acid.

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IDENTIFICATION OF γ -AMINOBUTYRIC ACID IN BRAIN BY THE ISOTOPE DERIVATIVE METHOD*

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There are many instances in which it is impossible to isolate sufficient quantities of an unknown substance from a mixture in order to identify it by such accepted methods as melting point and elementary analysis. When sufficiently sensitive analytical methods are available, identifications are often made at the microgram level from information such as distribution between solvents, R_F values obtained in chromatography, and spectral characteristics. However, identifications made by these methods alone often fail to distinguish between closely related substances. A procedure has recently come into wide use for identifying microgram quantities of substances in which the R_F values obtained on paper chromatograms are compared with those of a pure sample of the suspected substance. When such comparisons are made with several different solvents for developing the chromatograms, then one may assume that a reasonable identification has been made. Sometimes, however, such an assumption may not be valid. There are known cases in which two substances yield the same R_F values in many solvent systems. Among the amino acids, leucine and isoleucine yield but a single band on chromatograms, as do valine and norvaline. In such cases, final identification cannot be made by comparative paper chromatography. In a complex mixture, such as one might obtain from biological material, it is certainly unwise to depend solely upon comparative paper chromatography for identification.

The procedure with isotope derivative indicator, as it has been applied to the quantitative determination of amino acids (1), makes use of a technique that has the high sensitivity and specificity needed to provide rigorous identification at the microgram level. In this procedure, the amino acids in a mixture are converted to isotopic derivatives by reaction with I^{131} -labeled *p*-iodophenylsulfonyl chloride (pipsyl chloride). A measured amount of the S^{35} -labeled pipsyl derivative of the amino acid to be deter-

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mined is added to the mixture and the total sample is subjected to various purification procedures, followed by chromatography on paper. The chromatographic band of the substance under investigation is located by its S^{35} activity and cut into many transverse strips, and the ratio of I^{131} to S^{35} in each strip is measured. A constant ratio throughout the band indicates homogeneity and so identifies the I^{131} -labeled compound.

When one mixes and chromatographs two derivatives having R_f values close to one another, each derivative then forms a band displaced with respect to the other, the displacement being so small as to give the appearance of a single band. In such cases, inhomogeneity of the band can easily be detected by the mixed isotope procedure. This will be illustrated for the chromatograms of pipsylleucine and pipsylisoleucine and also for pipsylvaline and pipsylnorvaline.

We have applied this procedure to the identification of an amino acid isolated in partially purified form by paper chromatography from alcoholic extracts of mouse brain (2). This material showed identical chromatographic behavior with γ -aminobutyric acid in three different solvent systems. Previous reports (3, 4) of the finding of this amino acid in mammalian tissues were based solely on comparative paper chromatography. Since only microgram quantities can be obtained by this method, it would have required many chromatograms to isolate a sufficient quantity for identification by more rigorous methods. However, the small quantities that were obtained from a single chromatogram, several micrograms, were more than enough for identification by the procedure with isotope derivative indicator.

EXPERIMENTAL

The S^{35} -labeled pipsyl derivative of γ -aminobutyric acid was prepared by treating approximately 25 mg. of an authentic sample of the amino acid with S^{35} -labeled pipsyl chloride, according to the conditions described by Keston, Udenfriend, and Cannan (5). The purified derivative was dissolved in dilute ammonia. Approximately 0.6 γ of " γ -aminobutyric acid," representing about 10 per cent of a sample isolated from extracts of mouse brain by Roberts and Frankel (2) using paper chromatography, was treated with 10 mg. of I^{131} -labeled pipsyl chloride, according to the procedure previously described for quantitative pipsylation (5). After extraction of the acidified reaction mixture with ether, the combined ether extracts were extracted about eight times with twice the volume of 0.2 M HCl to remove *p*-iodophenylsulfonic acid. The ether was then evaporated and the residue taken up in about 0.2 ml. of ammoniacal alcohol (3 M ammonia in 50 per cent ethanol by volume).

Samples of each of the radioactive preparations (about 10,000 c.p.m.)

were transferred to strips of Whatman No. 1 filter paper. A mixture of the two preparations was also transferred to filter paper. The chromatograms were developed with *n*-butanol saturated with 1.0 N ammonia. The radioactive bands were located by radioautography and outlined with pencil. The individual bands were then cut into several transverse segments and each segment was eluted with water and transferred to a planchette, and the solution dried under an infra-red lamp. The elutions did not need to be quantitative, since all calculations are based on the ratio of one isotope to the other. Each sample was then measured with a thin window Geiger-Müller counter, a sufficient number of counts being taken to yield better than 1 per cent counting accuracy. A 0.003 inch aluminum absorber was then placed in front of the counter and the samples were counted again. Samples of pure I^{131} and pure S^{35} were also measured with and without the aluminum absorber. The aluminum absorber passes 40.2 per cent of pure I^{131} radiation (f_1) and 0.08 per cent of pure S^{35} radiation (f_2). A band composed of a mixture of the two isotopes would have a ratio of filtered to unfiltered counts somewhere between these two values. The proportion of each isotope in any sample may be calculated from the two simultaneous equations

$$\begin{aligned} I^{131} + S^{35} &= \text{counts without absorber} \\ (f_1)I^{131} + (f_2)S^{35} &= \text{counts with absorber} \end{aligned}$$

where I^{131} and S^{35} represent the number of counts due to the respective isotopes.

Samples of I^{131} -labeled pipsylisoleucine and S^{35} -labeled pipsylleucine, as well as I^{131} -labeled pipsylnorvaline and S^{35} -labeled pipsylvaline, were prepared, and mixed chromatograms of the two isomeric pairs were run as above.

Results

The sample obtained from brain yielded three bands on the chromatogram (Fig. 1). The major band, accounting for approximately 70 per cent of the I^{131} activity, coincided with the synthetic sample of pipsyl- γ -aminobutyric acid, and the proportion of the two isotopes was constant throughout. Two other bands appeared on the chromatogram. These contained only I^{131} and were probably valine and an unidentified peptide, as had been previously demonstrated (2).

In Fig. 2 there are presented the chromatograms of the pipsylleucines. Although the compounds move so closely together on the chromatogram that a mixture of the two yields a single band no wider than either of the components, it is evident from the changing isotopic proportions that the two compounds do not coincide exactly and are therefore not identical.

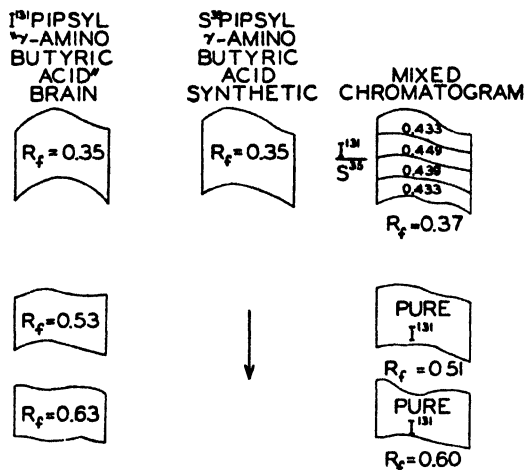
IDENTIFICATION OF γ -AMINO BUTYRIC ACID

FIG. 1. Chromatograms of the I^{131} -labeled pipsyl derivative obtained from extracts of mouse brain and the S^{35} -labeled pipsyl derivative of an authentic sample of γ -aminobutyric acid.

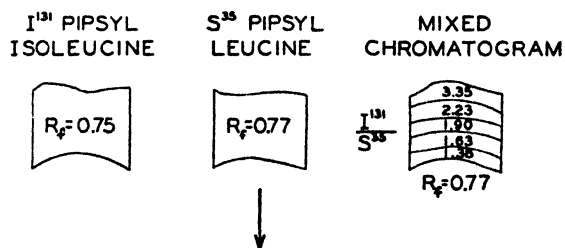


FIG. 2. Chromatograms of I^{131} -labeled pipsylisoleucine and S^{35} -labeled pipsylisoleucine.

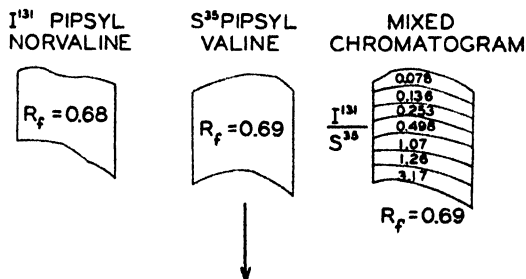


FIG. 3. Chromatograms of I^{131} -labeled pipsylnorvaline and S^{35} -pipsylvaline

In Fig. 3 is presented a similar experiment for the pipsylvalines. Here, too, a mixed chromatogram yielded a single band slightly broader than either of the individual compounds. However, an examination of the proportion of the two isotopes throughout the band indicated lack of constancy.

There are many obvious extensions of this technique to the identification of intermediates in metabolic experiments.

SUMMARY

1. Paper chromatography in conjunction with the two isotope techniques of labeling, as in the method with isotope derivative indicator, can be used as a rigorous method for chemical identification at the microgram level.

2. γ -Aminobutyric acid has been identified in extracts of mouse brain.

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THE METABOLIC FATE OF THE ISOPROPYL GROUP OF LEUCINE*

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Leucine is generally considered to be one of the most strongly ketogenic amino acids, in the sense that it yields an extra quantity of ketone bodies during the course of its catabolism in the diabetic organism or in isolated liver tissue. The mechanism of this transformation has recently been studied in this laboratory with the aid of radioactive carbon as a tracer (1). A preparation of the amino acid which was labeled in the β position with C^{14} was incubated with rat liver slices, and the resulting acetoacetate was degraded in order to establish the distribution of the radioactivity. Both the methyl and the methylene carbons were found to be labeled, indicating that at some stage in the metabolism of leucine its α - and β -carbons split off as a 2-carbon unit which is capable of condensing to form acetoacetic acid. The data were found to be consistent with the hypothesis that isovaleric acid is an intermediate in this series of reactions, but not with the assumption that isovalerate undergoes either reductive demethylation to form butyrate or oxidative demethylation to produce acetoacetate directly. In similar experiments in which γ - C^{14} -leucine was employed, the resulting acetoacetate was shown to be labeled in the carbonyl position only. This unexpected finding could not be explained adequately by any information then available on the metabolism of leucine in mammalian tissues.

A year ago, Zabin and Bloch (2) reported the formation of acetoacetate from isovaleric acid which was labeled with C^{13} and C^{14} . Their data are described as favoring a metabolic pathway which involves the splitting of the branched chain acid into a 2-carbon fragment and a 3-carbon fragment.

The studies on radioactive isovaleric acid which are described in the present paper were carried out in order to clarify the fate of the isopropyl group.¹ It has been possible to demonstrate that the 3 carbons under consideration are incorporated as a unit into the α , β , and γ positions of acetoacetic acid. Additional data have been obtained which indicate

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¹ A preliminary report of this investigation was presented at the meeting of the American Society of Biological Chemists at Atlantic City, April, 1950 (17).

that the carboxyl carbon of the acetoacetate which is formed in this manner may arise from carbon dioxide by a fixation reaction which has not been recognized previously. Accordingly, it is concluded that the complete breakdown of a mole of leucine in liver tissue leads to the formation of approximately 1.5 moles of ketone bodies.

EXPERIMENTAL

Determination of Radioactivity—Each of the compounds to be assayed for radioactivity was oxidized by the procedure of Van Slyke and Folch (3), and the resulting carbon dioxide was collected in a solution of barium hydroxide. The samples of washed and dried barium carbonate were counted according to procedures which have been described previously (1), employing a self quenching Geiger tube with a thin mica window. The counts per minute in excess of background were corrected to activity at infinite thickness and then expressed as counts per minute per mg. of carbon. By this procedure, a sample of barium carbonate exhibiting twice the radioactivity of the background would have a reported value of about 4. In the present paper, the recorded counts of 4 or greater are subject to a standard error not exceeding 5 per cent. The counts lower than 4 are subject to a greater error, and they are considered in most instances to be of questionable significance.

Synthesis of Carboxyl-Labeled Isovaleric Acid—Isobutyl alcohol which had been purified by fractional distillation was converted to the corresponding bromide by standard procedures (4). The Grignard reagent prepared from 7.4 gm. of the isobutyl bromide was allowed to react with radioactive carbon dioxide in an apparatus of the type described by Sakami, Evans, and Gurin (5). The resulting product was chilled overnight before it was decomposed to yield 1-C¹⁴-isovaleric acid. The yield of the acid after purification by distillation *in vacuo* was 65 per cent of the theoretical amount, based on the isobutyl bromide. The radioactivity of this preparation was 2390 c.p.m. per mg. of carbon, or 11,950 calculated for the carboxyl carbon alone.

Synthesis of β -Labeled Isovaleric Acid—Acetone with C¹⁴ in the carbonyl position was prepared by the pyrolysis of 1.8 gm. of carboxyl-labeled barium acetate in an evacuated, sealed tube heated to 530°. The product was collected in a receiving tube which was chilled in a chloroform-dry ice bath. The yield, according to an analysis of an aliquot portion by the gravimetric procedure of Van Slyke (6), was 74 per cent of the theoretical value. A sufficient amount of non-radioactive carrier was added to give a total of 1.44 gm. of acetone, which was then condensed with ethyl cyanoacetate in the presence of palladinized charcoal and hydrogen at a pressure of 30 pounds, according to the method of Alexander and Cope (7), thereby

producing ethyl isopropylcyanoacetate. The latter material was separated by distillation, and it was then heated under a reflux for 9 hours with 25 ml. of an aqueous 15 per cent solution of sodium hydroxide. Acidification of the reaction mixture yielded isopropylmalonic acid, which was separated and then decarboxylated by heating the dry acid for 30 minutes in a dry flask immersed in an oil bath at 200°. The resulting isovaleric acid, purified by distillation *in vacuo*, was obtained in a yield of 51 per cent of the theoretical amount as calculated from the acetone. The product had 1090 c.p.m. per mg. of carbon, or 5450 calculated for the β -carbon alone.

Synthesis of Methyl-Labeled Isovaleric Acid—The general procedures which are outlined in the preceding paragraph were used in converting 2-C¹⁴-barium acetate into methyl-labeled acetone, and in transforming the latter substance into γ, γ' -C¹⁴-isovaleric acid. The final product had a radioactivity of 4940 c.p.m. per mg. of carbon, or 12,350 calculated for each of the methyl carbons.

Incubation of Substrates with Tissue Slices—In general, the procedures previously employed in the work with radioactive leucine were followed in the present studies. A solution of 2.0 mg. of sodium isovalerate in 20 ml. of Ringer-phosphate buffer solution was added to each Warburg flask, and 2.5 gm. (moist weight) of rat liver slices were then introduced. Oxygen was employed as the gas phase, and a 4 hour incubation at 38° was carried out. The solution of alkali in the center wells of four such flasks was then removed with a capillary dropping pipette and stored under toluene until it was convenient to liberate the carbon dioxide and aerate it into a saturated solution of barium hydroxide. The combined incubation medium from the same four flasks was then treated with the copper-lime reagents as used by Edson (8) to accomplish deproteinization. At the end of half an hour the mixture was centrifuged. To the water-clear supernatant solution, which contained on the average 16 mg. of acetoacetate, non-radioactive acetoacetate (9) was added as a carrier.

Degradation Procedures—Aniline citrate and Denigès' reagent were used according to the directions of Edson (8) to decompose the acetoacetate. The mercury-acetone precipitates were removed from the hot solutions as recommended by Cohen (10), in order to avoid possible contamination by other substances which might form insoluble complexes in the cold. Invariably, these precipitates were purified by treatment with hydrochloric acid and by steam distillation of the liberated acetone into fresh mercuric sulfate reagent. In certain instances when it was desired to prepare iodoform, the acetone was distilled *in vacuo* into a chilled centrifuge tube containing 3.0 ml. of 15 N sodium hydroxide, and 5 ml. of an iodine solution (11) were added. At the end of half an hour the crystalline iodoform was collected by centrifugation, and it was washed thoroughly with distilled

water until the washings were free of alkali. The yields of iodoform were in the neighborhood of 96 per cent, and the melting points of these samples gave no indication of the presence of impurities.

In some experiments, the permanganate degradation method of Weinhouse and Millington (12) was employed. The formic acid obtained by this procedure was oxidized to carbon dioxide by the action of mercuric sulfate, and the acetic acid was then identified by the Duclaux constants before it was isolated as the barium salt. The salt was pyrolyzed as outlined previously (1) in order to determine experimentally the distribution of radioactivity between its carbons.

TABLE I

Distribution of Radioactivity in Acetoacetate Formed from Carboxyl-Labeled Isovaleric Acid in Liver Slices

Method of degradation*	Compound	Carbons of acetoacetic acid represented	C.p.m. per mg. of carbon	
			Experiment 1	Experiment 2
Denigès' reagent, heat	Carbon dioxide	COOH	146	102
Aniline citrate	" "	"		104
Denigès' reagent, heat	Acetone	CH ₃ , CO, CH ₂	36	30
Heat	"	" " "		31
	Iodoform	" CH ₂		0
		CO (calculated)	108	92

* Prior to the degradations, 90 mg. of carrier acetoacetate were added in Experiment 1, and 104 mg. in Experiment 2.

In one of the experiments to be described, β -hydroxybutyrate was converted to acetoacetate and then degraded according to well established methods (13).

Results

Fate of 1-C¹⁴-Isovalerate in Liver Slices—On the basis of the previous work with radioactive leucine, one would expect that carbons 1 and 2 of carboxyl-labeled isovalerate would furnish doubly labeled acetoacetate in liver tissue. The data recorded in Table I support this view, for in each of the experiments the tracer was found in both the carbon dioxide and the acetone fractions. The radioactivity of the acetone has been calculated as being present in the carbonyl carbon only. It would seem extremely unlikely that any appreciable amount of the labeled carbon might have found its way into the methyl or methylene positions of the acetoacetate, but in order to be certain of this point, iodoform was prepared in Experiment 2. As expected, there was no measurable radioactivity.

To check the reliability of the carboxyl sample obtained by heat decarboxylation, a separate aliquot of the copper-lime filtrate was submitted to the aniline citrate degradation in the second experiment. The values obtained by these two independent methods are in good agreement. The count of the acetone sample, which was prepared in the usual fashion by the addition of Denigès' reagent to the acetoacetate solution, was also checked in the second experiment by carrying out the decarboxylation with heat, and then steam distilling the acetone into Denigès' reagent. Here again the values are in accord, giving additional assurance that the degradation procedures gave reliable results.

It is of interest that the ratio of carboxyl to carbonyl radioactivity in the acetoacetate is slightly greater than unity in each case; the values are 1.4 and 1.1, respectively. These ratios, along with similar values previously obtained in the β -C¹⁴-leucine work, make it appear likely that the 2-carbon

TABLE II
Recovery of Radioactivity in Studies with Carboxyl-Labeled Isovalerate

Compound	Experiment 1		Experiment 2		Per cent recovery, average
	Total c.p.m.	Per cent recovery	Total c.p.m.	Per cent recovery	
Administered sodium isovalerate...	9250		9250		
Respiratory CO ₂	470	5	950	10	8
Acetoacetic acid	3170	34	2760	30	32

unit under discussion here is identical with that arising from the first 2 carbons of straight chain fatty acids (14-16).

The recovery of the administered C¹⁴ is summarized in Table II. The total counts in the respiratory carbon dioxide were calculated from the amount and the radioactivity of the barium carbonate which it yielded. The total counts in the acetoacetate were calculated from the amount of this material, including carrier, known to be present in the copper-lime filtrates, and from the radioactivity of the carbon dioxide and the acetone fractions produced in the standard Denigès' degradation. As may be seen from the data, about one-third of the administered counts was recovered in the acetoacetate. The respiratory carbon dioxide also had appreciable radioactivity, indicating that a portion of the 2-carbon units was completely oxidized, presumably via the tricarboxylic acid cycle.

Fate of β -C¹⁴-Isovalerate in Liver Slices—The results presented in Table III show that isovalerate having a label in the center carbon of its isopropyl group gives rise to acetoacetate with radioactivity primarily in the carbonyl position, a finding which is in complete accord with the previous

work on γ - C^{14} -leucine. Although one would hardly expect to find an appreciable amount of the C^{14} in the methyl or methylene carbons, this possibility was investigated in Experiment 2 by the preparation of iodoform. As anticipated, radioactivity could not be detected in this compound. In each experiment, therefore, the carbonyl carbon was calculated as containing all of the radioactivity of the acetone. For additional indirect proof that essentially mono-labeled acetoacetate was produced in these experiments, the β -hydroxybutyrate was also degraded in Experiment 3. The

TABLE III

Distribution of Radioactivity in Acetoacetate and β -Hydroxybutyrate Formed from β -Labeled Isovaleric Acid in Liver Slices

Method of degradation*	Compound	Carbons of acetoacetate or β hydroxybutyrate represented	C p m. per mg. of carbon		
			Ex- peri- ment 1	Ex- peri- ment 2†	Ex- peri- ment 3‡
Denigès' reagent, heat " " "	Carbon dioxide	COOH	6	4	9
	Acetone	CH ₃ , CO, CH ₂	48	38	86
	CHI ₃	" CH ₂		0	
		CO (calculated)	144	114	258
Dichromate, Denigès' re- agent, heat " "	Carbon dioxide	COOH			8
	Acetone	CH ₃ , CHOH, CH ₂			54
		CHOH (calculated)			162

* Prior to the acetoacetate degradations, 90 mg. of carrier acetoacetate were added in Experiment 1, 83 mg. in Experiment 2, and 45 mg. in Experiment 3.

† 2 mg. of substrate were incubated in each of three flasks.

‡ Prior to the hydroxybutyrate degradation in Experiment 3, 22 mg. of carrier acetoacetate were added.

acetone fraction from the latter compound contained most of the C^{14} , and the radioactivity of the β -carbon was calculated to be 20 times that of the carboxyl carbon. The data on the recovery of the administered radioactivity are summarized in Table IV. An average of 44 per cent of the administered counts was recovered in the acetoacetic acid, but only 2 per cent was accounted for in the respiratory carbon dioxide.

In explanation of these findings, it was tentatively proposed that the isopropyl group of leucine (or isovalerate) yields a 2-carbon intermediate which acts almost exclusively as an acetylating agent in forming acetoacetate (17). A similar hypothesis has been used by Crandall, Brady, and Gurin (18) in order to explain the behavior of the terminal fragment arising from octanoic acid. These investigators incubated β -labeled oc-

tanoate with washed homogenates of rat liver, and found that the resulting acetoacetate contained about 3 times as much labeled carbon in the carbonyl position as was in the carboxyl position.

Fate of γ, γ' -C¹⁴-Isovalerate in Liver Slices—In order to put to further test the theory of a strongly acetylating fragment arising from the isopropyl group, the metabolism of isovalerate with C¹⁴ in the methyl groups

TABLE IV
Recovery of Radioactivity in Studies with β -Labeled Isovalerate

Compound	Experiment 1		Experiment 2		Experiment 3		Per cent recovery, average
	Total c.p.m.	Per cent recovery	Total c.p.m.	Per cent recovery	Total c.p.m.	Per cent recovery	
Administered sodium isovalerate.	4220		3160		4220		
Respiratory CO ₂	90	2	80	3	60	1	2
Acetoacetic acid	1870	44	1320	42	1920	45	44

TABLE V
Distribution of Radioactivity in Acetoacetate Formed from Methyl-Labeled Isovaleric Acid in Liver Slices

Method of degradation*	Compound	Carbons of acetoacetic acid represented	C.p.m. per mg. of carbon	
			Experiment 1	Experiment 2
Denigès' reagent, heat	Carbon dioxide	COOH	7	9
" " "	Acetone	CH ₃ , CO, CH ₂	202	382
Permanganate	Formic acid	CH ₂	249	446
Pyrolysis of acetate	BaCO ₃	CO	2	2
	Acetone	CH ₃ , CO, CH ₂	203	276
		CH ₃ (calculated)	304	413

* Prior to the degradations, 90 mg. of carrier acetoacetate were added in Experiment 1, and 43 mg. in Experiment 2.

was investigated. As may be seen in Table V, radioactivity was found to be concentrated in the methylene and methyl carbons of the resulting acetoacetate, and to approximately the same extent in each position. The ratios of methylene to methyl radioactivity are 0.8 and 1.1 in the two experiments. Obviously, it may be concluded that all 3 carbons of the isopropyl group are involved in acetoacetate formation, and as a consequence the hypothesis of the formation of a highly acetylating 2-carbon fragment from the isopropyl group may now be discarded.

It should be noted that the radioactivity as measured by the perman-

ganate degradation in Experiment 1 accounts for 92 per cent of the activity of the mercury-acetone precipitate. A similar calculation in Experiment 2 gives a figure of 75 per cent. Acetoacetate is known to decarboxylate slowly upon standing in copper-lime filtrates, and since the permanganate procedure, unlike the Denigès' reagent, does not attack acetone, one would expect the former procedure to detect somewhat less radioactivity in these experiments in which dilution by carrier must be taken into account. The satisfactory agreement of the data obtained by these two independent degradative procedures therefore justifies the conclusion that the 3 carbons of the isopropyl group were incorporated into the α , β , and γ positions of the acetoacetate.

Further support for this view is obtained from the data in Table VI on the recovery of radioactivity in the acetoacetate and in the respiratory

TABLE VI
Recovery of Radioactivity in Studies with Methyl-Labeled Isovalerate

Compound	Experiment 1		Experiment 2		Per cent recovery, average
	Total c.p.m.	Per cent recovery	Total c.p.m.	Per cent recovery	
Administered sodium isovalerate ..	19,120		19,120		
Respiratory CO ₂	270	1	160	1	1
Acetoacetic acid	7,640	40	8,020	42	41

carbon dioxide. The distribution is, within experimental error, the same as that which was found when β -C¹⁴-isovalerate was employed.

Studies with Radioactive Bicarbonate in Liver Slices—The incorporation of the isopropyl group of isovalerate into acetoacetic acid raises the question of the origin of the carboxyl carbon of the latter compound. The possibility that carbon dioxide might play a rôle in this reaction was investigated by the incubation of C¹⁴-labeled sodium bicarbonate with liver slices in the presence of added non-radioactive leucine or isovaleric acid. In each experiment listed in Table VII, the recorded amount of substrate was divided equally between two flasks, each containing 0.05 mm of sodium bicarbonate (having approximately 20,000 c.p.m. per mg. of carbon), 20 ml. of Ringer-phosphate buffer solution, and 2.5 gm. of liver slices. One-half of the copper-lime filtrate from each experiment was heated under a reflux with Denigès' reagent in order to obtain the mercury-acetone complex. In no instance did this acetone fraction contain an appreciable amount of the administered radioactivity.

The remaining half of the acetoacetate solution was treated with aniline citrate after precautions had been taken to dilute and remove any dis-

solved radioactive carbon dioxide. To accomplish this, 1 gm. of non-radioactive sodium bicarbonate was dissolved in the solution, dilute sulfuric acid was added to about pH 4, and carbon dioxide-free air was then bubbled through rapidly for 45 minutes. This procedure was repeated twice before the decarboxylation was carried out. The results presented in Table VII clearly indicate that radioactive carbon dioxide was fixed during the metabolism of added isovaleric acid or DL-leucine, whereas there was no appreciable fixation in the absence of these substrates.

TABLE VII
Role of Carbon Dioxide in Leucine Catabolism

Experiment No.	Substrate	Amount of substrate added	C.p.m. per mg. of carbon*	
			Mercury-acetone complex	CO ₂
1	Isovaleric acid	0.08	1	145
2	" "	0.08	0	111
3	" "	0.08		166
4	" "	0.02		29
5	DL-Leucine	0.16	0	35
6	"	0.16	1	44
7	None		0	1
8	"		0	2

* Prior to the degradations, 54 mg. of carrier acetoacetate were added in each experiment.

DISCUSSION

As a result of the work of many investigators, it is now recognized that the major pathway for acetoacetate formation in the liver is the condensation of C₂ units derived from straight chain fatty acids or from pyruvate. It is also known that the 4 carbons of acetoacetate may arise as an intact unit during the breakdown of phenylalanine and tyrosine (12, 19-21). The evidence in the present paper for a C₃-C₁ condensation is apparently the first recorded instance of a third type of pathway leading to the formation of ketone bodies in liver tissue.

The series of reactions in Diagram 1 illustrates the findings with the three different varieties of radioactive isovalerate. The 2-carbon compound which forms doubly labeled acetoacetate is shown as acetic acid, with the understanding that it may be identical with "active acetate" arising from straight chain fatty acids. In the event that the splitting of the carbon chain of isovalerate involves β -oxidation, one would expect some oxygen-containing group to be present at the center carbon of the 3-carbon

unit which is shown in brackets. This fragment is shown as undergoing direct carboxylation to produce acetoacetate, although some more complicated mechanism cannot, of course, be entirely ruled out at the present time. It would appear that the inability of Swendseid, Barnes, Hemingway, and Nier (22) to show the incorporation of C^{13} -labeled sodium bicarbonate into the urinary ketone bodies of fasted rats was due to the high biological dilutions inherent in their experiments.

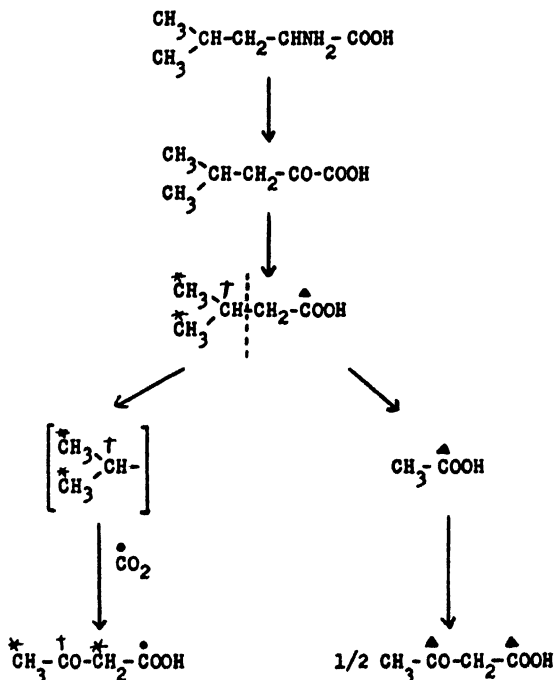


DIAGRAM 1. Proposed mechanism for the conversion of leucine to acetoacetate

The 3-carbon intermediate most likely is not a glycogenic substance, for a variety of investigators have been unable to show the formation of extra carbohydrate from leucine or isovalerate. Pyruvate, in particular, can be eliminated from consideration because in liver preparations it forms acetoacetate by a route involving the condensation of C_2 units. Preliminary studies in this laboratory² indicate that the incubation of acetone with liver slices results in the fixation of a slight but significant amount of radioactive carbon dioxide in the carboxyl position of acetoacetate.³ Borek

² Coon, M. J., unpublished data.

³ In a private communication, Dr. G. W. E. Plaut states that he and Dr. H. A. Lardy have also obtained experimental evidence for the incorporation of the car-

and Rittenberg (23) have demonstrated the formation of deuteriocholesterol from deuterioacetone in surviving rat liver slices, and more recently Sakami (24) has reported evidence for the production of "formate" from the methyl groups of acetone in the intact rat. It is apparent that acetone need no longer be considered strictly as an end-product of mammalian metabolism, but further investigation will be required before a final decision can be reached as to the rôle of this compound in the conversion of leucine to acetoacetate.

SUMMARY

1. Studies which have recently been carried out on the metabolism of radioactive isovaleric acid in liver slices are in complete accord with earlier findings obtained with radioactive leucine, thereby confirming the belief that the former compound is an intermediate in the breakdown of the amino acid.

2. Carbons 1 and 2 of isovalerate furnish a 2-carbon intermediate which is capable of condensing to form acetoacetate.

3. The 3 carbons of the isopropyl group of isovalerate are incorporated as a unit into the α , β , and γ positions of acetoacetic acid. The carboxyl carbon of the acetoacetate which is produced in this manner arises from carbon dioxide by a fixation reaction which has not been recognized previously.

4. It may be concluded that the complete catabolism of a mole of leucine in liver tissue leads to the formation of approximately 1.5 moles of ketone bodies.

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INFRA-RED ABSORPTION SPECTRA OF TOCOPHEROLS AND SOME OF THEIR CHEMICAL PRODUCTS*

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In a previous report (1) the usefulness of infra-red absorption spectra in differentiating the tocopherols was demonstrated. The present investigations represent an extension of these earlier studies to certain related compounds and to some of the oxidation products of the tocopherols. Other workers have published infra-red absorption data on the tocopherols (2) and on a few of the oxidation intermediates (3). A portion of the absorption spectrum of α -tocopherylhydroquinone was reported on recently (4). However, practically all of the previous reports give data over only relatively short ranges of the absorption spectra. Therefore, it appears desirable to report not only information on compounds not heretofore studied, but also more complete infra-red absorption spectra on previously studied compounds, when such additional data are considered to be important. All the published data in addition to this report should result in a significant catalogue of infra-red absorption spectra of vitamin E substances and some of their chemical products. Such a catalogue undoubtedly will be valuable in the identification of the intermediates produced in tocopherol reaction mixtures. Moreover, infra-red analysis can be useful in establishing the relative purity of the tocopherols and related compounds intended for biologic assay.

Methods

The infra-red absorption spectra were obtained on a Baird Associates (5) infra-red recording spectrophotometer.¹ The spectral range covered was from 2 to 16 μ . The substances were investigated either as oil smears or as melts between two rock salt plates. The oxidation products of the tocopherols were prepared according to methods in the literature (6) or devised in this laboratory.^{2, 3}

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¹ We wish to express our grateful appreciation to Dr. James D. Hardy and the Department of Physiology of Cornell University Medical College for permission to use this instrument.

² Ulick, S., Rosenkrantz, H., and Milhorat, A. T., unpublished data.

³ Rosenkrantz, H., Ulick, S., and Milhorat, A. T., unpublished data.

The sample of tocopherylhydroquinone was prepared cautiously because of its susceptibility to molecular oxidation. A purified sample of this compound was dried under high vacuum for 24 hours previous to infra-red analysis. The dried material was melted between two plates of sodium chloride under nitrogen and studied immediately. One sample remained a glass-like oil, but a second sample crystallized during the period of observation; however, the spectra were identical.

EXPERIMENTAL

The absorption spectra shown here are copies of original automatic recordings. Corrections in calibration have been made only to align the absorption band near 3.4μ in all the curves. The absorption curves of the following substances were obtained: natural β -tocopherol, synthetic ϵ -tocopherol, *dl*- α -tocopheramine, β -, γ -, and δ -tocopherylquinone, α -tocopherylhydroquinone, and α -tocopherylhydroquinone diacetate.⁴

Analysis of Spectra

Since certain atomic groups specifically absorb in relatively well known regions of the infra-red, they will be discussed first.

O—H and N—H₂ Absorption—All the tocopherols studied here have a hydroxyl group which absorbed in the 3μ region. Since the earlier work (1) did not distinguish hydroxyl groups of alcoholic type from those of phenolic type, this region was examined more closely. Because of the greater resolution of the new instrument, observations in the 3μ region could be partially clarified. The results are shown in Table I.

It can be seen from Table I that the absorption maxima of the tertiary alcoholic hydroxyl groups of the quinones appear at a lower wave-length than the absorption maxima of the phenolic hydroxyls. This was to be expected, since alcoholic hydroxyl groups absorb at lower wave-lengths (7). The difference in the location of hydroxyl absorption did not appear to be significant when the quinones and α -tocopherol were compared, but was more evident when comparison of the quinones and the partially or completely demethylated tocopherols (δ - and ϵ -tocopherol) was made.

The progressive shift to higher wave-lengths of the absorption of the hydroxyl groups of α -, β -, γ -, and δ -tocopherol suggested an effect on the hydroxyl vibrations by neighboring methyl groups (Fig. 1). However, the single methyl group of δ -tocopherol appears to have no such effect,

⁴ We wish to acknowledge our gratitude to the following for some of the pure samples of tocopherol substances studied in this work: Dr. J. G. Baxter of Distillation Products, Inc., Rochester, New York, for β - and ϵ -tocopherol, and Dr. B. Tischler of Merck and Company, Inc., Rahway, New Jersey, for α -tocopheramine hydrochloride.

TABLE I
O—H and N—H₂ Absorption in 3 μ Region

Compound	Chemical group	Wave-length
α -Tocopherol	Phenolic O—H	2.97
β -Tocopherol	" "	3.01
γ -Tocopherol	" "	3.01
δ -Tocopherol	" "	3.05
ϵ -Tocopherol	" "	3.05
α -Tocopherylquinone	Alcoholic O—H	2.96
β -Tocopherylquinone	" "	2.96
γ -Tocopherylquinone	" "	2.96
δ -Tocopherylquinone	" "	2.96
α -Tocopherylhydroquinone	Phenolic " } Alcoholic "	3.10
α -Tocopheramine	N—H ₂	{ 2.97 3.01
α -Tocopherylhydroquinone diacetate	Alcoholic O—H	2.96

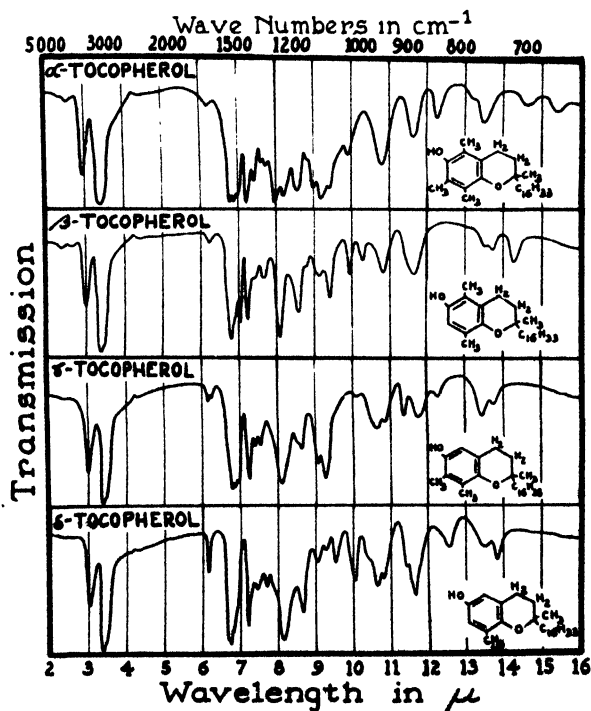


FIG. 1

since the hydroxyl absorption of this tocopherol and that of the unmethylated compound, ϵ -tocopherol, were identical. Moreover, since the hydroxyl absorption in these two compounds was near 3.05μ , a value usually considered high for unbonded hydroxyls, the possibility of intermolecular hydrogen bonding was suggested (8). This suggested further that methyl groups situated near the hydroxyl group in the tocopherols impede hydrogen bonding, probably through steric hindrance (9). This effect was seen to be pronounced in α -tocopherol which has methyl radicals on either side of the hydroxyl group.

This suggested a plausible explanation of the difficulty encountered in distinguishing the phenolic hydroxyl group in α -tocopherol from the tertiary alcoholic hydroxyl groups in the tocopherylquinones and in the tocopherylhydroquinone diacetate. Internal chelation involving the oxygen of the broken pyran ring and the hydrogen of the tertiary alcohol could shift the absorption of the hydroxyl in the quinones to a longer wavelength. This phenomenon in conjunction with the hindrance to bonding by the methyl groups in α -tocopherol could conceivably result in the appearance of the hydroxyl absorption of both substances at the same frequency. Additional evidence for the occurrence of chelation in the quinones will be referred to in the discussion on carbonyl absorption.

It was too difficult to establish the effect of the methyl groups of α -tocopherylhydroquinone on hydrogen bonding, since it was apparent that the forces of hydrogen bonding existing in this molecule were greater than those in the tocopherols. This was revealed by the occurrence of the hydroxyl absorption near 3.10μ (4) and by the shape of the hydroxyl absorption band (Fig. 2). Hydroxyl bands that tend to broaden and merge with the nearby C—H band usually suggest strong hydrogen bonding (7). Two possibilities of bonding, namely internal chelation and intermolecular hydrogen bonding, existed in the tocopherylhydroquinone molecule (Fig. 3).

In the case of internal chelation a 7-membered ring could form. This possibility seemed attractive because the spectral region from 9 to 16μ of the tocopherylhydroquinone appeared similar to its tocopherol but not to its quinone. It is conceivable that chelation caused a partial reconstruction of the absorption characteristics of the chroman nucleus. Moreover, some intermolecular hydrogen bonding possibly would occur, as in the case of the tocopherols.

The N—H₂ absorption of α -tocopheramine also occurred in the 3μ region. The double band near 3μ was assigned to the amino vibrations. The intensity of the absorption bands of the amino group appeared to be remarkably weak, as did some of the bands at longer wave-lengths (Fig. 4). In the case of tocopheramine, this may have been due partially to the fact that smaller amounts were used in the analysis. However, one would have

anticipated more intense absorption bands, particularly those due to the amino group, since the C—H band at $3.41\ \mu$ seemed moderately intense.

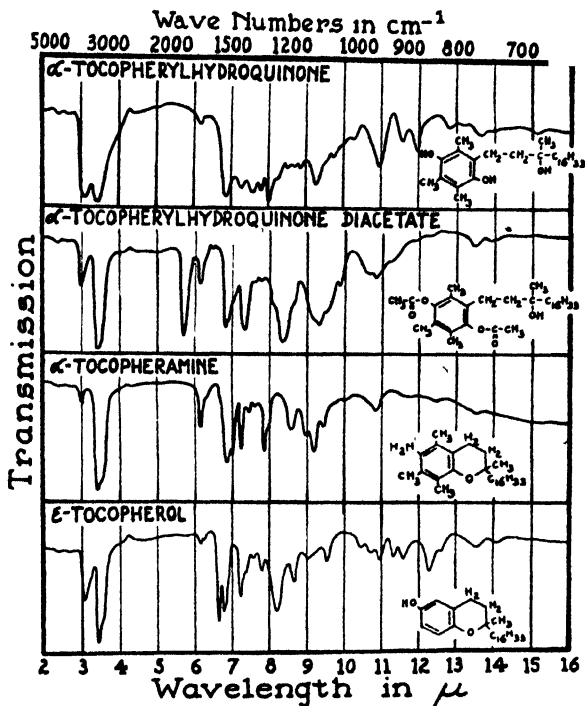
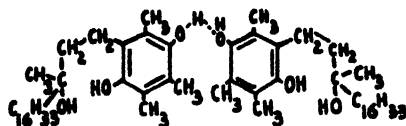
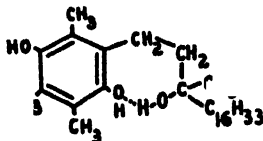


FIG. 2



A. INTERMOLECULAR HYDROGEN BONDING



B. INTERNAL CHELATION

FIG. 3

The amino absorption doublet of significant intensity had been observed in veratrole compounds.⁵ Since the NH_2 absorption occurred at a longer

⁵ Furchgott, R. F., and Rosenkrantz, H., unpublished data.

wave-length than normally expected (10), it appeared that this chemical group also was involved in hydrogen bonding (10). The amino absorption doublet has been interpreted as being due to two vibrations, one in which both hydrogen atoms simultaneously approach the nitrogen atom and another involving alternate oscillations of the hydrogen atoms toward the nitrogen atom (7).

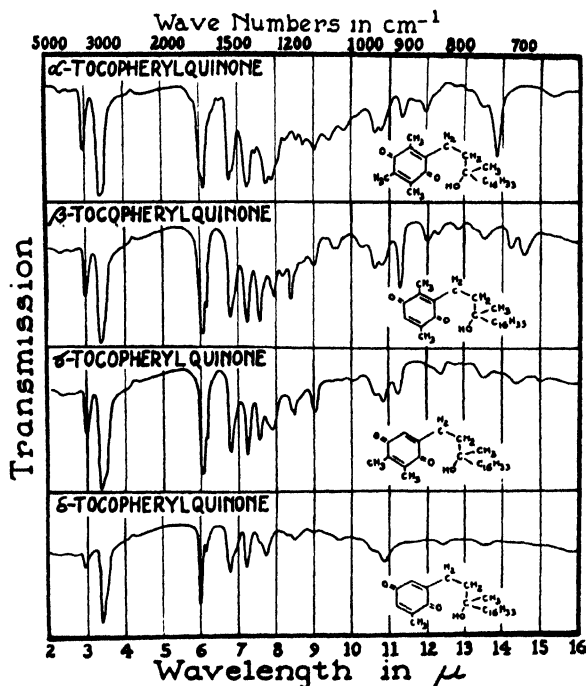


FIG. 4

C—H and CH₃ Absorption—All the compounds studied gave strong absorption bands near 3.4 μ and between 6.6 and 7.1 μ . These are assigned to the various C—H vibrations from the C—H, C—H₂, and C—H₃ groups of both aromatic and aliphatic types (7).

Most of these substances have one or more CH₃ groups on the benzene ring and several on the phytyl side chain. The CH₃ vibration was referred to the strong band near 7.2 μ (7).

C=O and C=C Absorption—In the previous paper (1), it was demonstrated that benzoquinones gave a strong C=O absorption band near 6.0 μ and a C=C band near 6.3 μ . Corresponding absorption bands in the spectrum of α -tocopherylquinone were assigned accordingly. In the present work it was found that these absorption bands were not always resolved

as two distinct bands. Several observations on α -tocopherylquinone disclosed that the C=O and the C=C bands sometimes occurred near 6.02 and 6.10 μ , respectively. In other cases, these bands apparently merged to give an absorption band near 6.08 μ .

The C=O and C=C absorption bands of β -, γ -, and δ -tocopherylquinones were resolved into two components. In β -tocopherylquinone, these bands were near 6.07 and 6.18 μ , in γ -tocopherylquinone near 6.05 and 6.18 μ , and in δ -tocopherylquinone near 6.02 and 6.18 μ , respectively. In every case, the absorption near 6 μ was extremely intense, while that near 6.2 μ was moderately weak.

A comparison of these observations with those made earlier on benzoquinone and naphthoquinone (1) suggested that hydrogen bonding may be affecting the absorption in this region. Both chelation and intermolecular hydrogen bonding could occur in the tocopherylquinones (Fig. 3). If bonding occurred, the effect seemed quite small, as the carbonyl absorption (11) shifted only from about 6.03 μ (the value for a quinone C=O) to about 6.08 μ in α -tocopherylquinone. A more significant change was the tendency of the C=C absorption band to diminish and probably separate from the C=O band as one proceeded from α - to δ -tocopherylquinone (Fig. 4).

The conjugated C=C absorption band of the benzene ring in the tocopherols and in tocopherylhydroquinone was near 6.2 μ . This band was of weak to moderate intensity, and sometimes occurred as a doublet.

The C=O absorption of the ester groups in α -tocopherylhydroquinone diacetate occurred near 5.72 μ , as observed in the corresponding triacetate (1) (Fig. 2).

Phenolic C—O and C—N Absorption—The C—O absorption occurred near 8 μ as previously indicated (1). Because of the nature of the broad absorption band recorded on the older instrument, some discrepancy in wave-length has resulted. However, the effect of the methyl groups on the C—O vibrations was still significant. As one proceeded from α - to ϵ -tocopherol, the wave-length of the C—O absorption was 7.95, 8.10, 8.12, 8.17, and 8.21 μ , for α -, β -, γ -, δ -, and ϵ -tocopherol, respectively.

Studies on α -tocopheramine revealed an absorption band near 7.88 μ with a side inflection near 7.95 μ . The band near 7.95 μ probably was due to the C—O group containing the oxygen from the pyran ring. The band near 7.88 μ was assigned to the C—N vibrations. Carbon to nitrogen vibrations of this type usually occur at shorter wave-lengths than the corresponding carbon to oxygen vibrations (7).

It would appear that the C—O absorption in α -tocopherylhydroquinone diacetate was shifted to 8.35 μ , due probably to influence of the ester groupings, since the corresponding absorption in α -tocopherylhydroquinone was near 8.0 μ .

Other Absorption Bands—There appeared to be some relationship between certain infra-red absorption bands and the presence of an intact chroman nucleus. All the tocopherols gave significant absorption near 8.6, 9.3, and 11.6 μ , while the corresponding quinones absorbed near 7.7, 7.9, 10.6, and 11.4 μ . These absorption bands could possibly be used for differentiating the chroman structure from the quinone configuration.

As mentioned previously, the absorption spectrum of α -tocopherylhydroquinone resembles that of its tocopherol more closely than it does the spectrum of its quinone. Absorption was observed near 9.3 and 11.6 μ .

Certain bands appeared to be characteristic of the tocopherol molecule as reported previously (1). Absorption near 10.8, 12.2, and 13.5 μ was observed in all of the tocopheryl compounds.

It may be concluded that the presence of certain characteristic absorption bands permits differentiation among all the tocopherol substances thus far studied. Some of the compounds have one or more unique bands which allow immediate and positive identification (*e.g.* the 13.95 μ band in α -tocopherylquinone).

SUMMARY

1. The infra-red absorption spectra, from 2 to 16 μ , of the known tocopherols and of some of their chemical products have been presented.
2. An analysis of certain absorption bands in relation to structural configuration was attempted.
3. Characteristic bands were described for differentiating the tocopherols from their chemical products.
4. The effect of hydrogen bonding was observed in most of the substances and was particularly outstanding in α -tocopherylhydroquinone.

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A MODIFIED METHOD OF ESTIMATION OF XANTHINE OXIDASE IN TISSUE HOMOGENATES*

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In the course of testing the effect of diet on rat liver xanthine oxidase by the method of Axelrod and Elvehjem (1) some anomalous results were obtained. A number of "zero" livers similar to those recently described by Richert, Edwards, and Westerfeld (2) were found. In this determination xanthine oxidase activity is calculated as the difference between the endogenous oxygen uptake and endogenous plus xanthine oxidase oxygen uptake. The cause of the zero titers was attributed to the inhibitory effect of an excess of substrate added to a small amount of enzyme which was already saturated with endogenous substrate. After the removal of endogenous substrate from the homogenate by dialysis, a positive titer was demonstrable. Inasmuch as dialysis is an inconvenient procedure to employ, it occurred to us that we could exclude oxygen uptake due to the oxidation of endogenous purines by xanthine oxidase from the endogenous respiration by the addition of the highly potent inhibitor, 2-amino-4-hydroxy-6-pteridinecarboxaldehyde (AHPC). This we have used as the basis for an improved method for the estimation of xanthine oxidase in rat liver homogenates.

EXPERIMENTAL

Albino rats (160 to 190 gm.) were used for the experiments. Half of these were fed a synthetic casein diet (Diet R33) and the remainder was fed a fortified natural diet (Diet R58A) for about 6 weeks before use. Diet R33 is that of Spicer *et al.* (3). The per cent composition of Diet R58A is as follows: dextrose 18, yellow corn-meal 30, whole wheat meal 10, soy bean meal 25, skim milk powder 10, dried liver cake 3, brewers' yeast 1, bone meal 1, iodized salt 1, riboflavin 0.0005, thiamine hydrochloride 0.0003, pyridoxine hydrochloride 0.0005, calcium pantothenate 0.002, niacinamide 0.001, choline chloride 0.1, vitamin A 8750 i.u. per kilo, vitamin D 1800 i.u. per kilo, vitamin E 0.000025, and vitamin K 0.00125.

The liver homogenates were prepared in the following manner: (a) the rat was decapitated and thoroughly bled; (b) the liver quickly removed,

* A preliminary report was presented at the meetings of the Federation of American Societies for Experimental Biology, 1950 (*Federation Proc.*, 9, 241 (1950)).

blotted on paper, and weighed on a tared watch-glass; (c) the liver was placed in a small Waring blender with 5 volumes of 0.039 M phosphate buffer (pH 7.4) containing equal concentrations of sodium and potassium ions and was homogenized for exactly 3 minutes.

1.5 ml. of homogenate (equivalent to 250 mg. of liver) were placed in respiration flasks containing appropriate materials. Four flasks were used for each rat liver homogenate. The first contained homogenate, to the second was added in addition 30 γ of 2-amino-4-hydroxy-6-pteridine-carboxaldehyde, the third contained 0.2 ml. of 0.05 M hypoxanthine in the side arm, and the fourth 0.2 ml. of 0.05 M xanthine in the side arm. The center well in all flasks contained 0.2 ml. of 10 per cent potassium hydroxide. Sufficient water was added to each flask to make a total volume of 3 ml. The flasks were attached to manometers and placed in a thermostat set at $38^{\circ} \pm 0.05^{\circ}$. After 10 minutes equilibration, the manometers were set and readings taken every 20 minutes thereafter for 140 minutes. After 50 minutes incubation the substrate was added. Successive rat livers were assayed at 20 minute intervals to maintain a constant time interval between decapitation and incubation. It has been found that the addition of substrate to the flask containing inhibitor (AHPC) at the level used (120 γ per gm. of liver) does not alter the oxygen uptake. We are indebted to Dr. C. W. Waller for the 2-amino-4-hydroxy-6-pteridinecarboxaldehyde which was prepared by a new method (4).

Results

Table I shows the mean oxygen uptake of rat liver per hour due to xanthine oxidase after the addition of substrate (either hypoxanthine or xanthine) with or without inhibitor (AHPC). Two different diets were purposely used to produce a wide range of titers in order that the different treatments might be evaluated for general applicability. The data in Table I are representative samples taken from a much larger body of unpublished work on the effect of diet on liver xanthine oxidase. The analyses of variance of these data, shown in Table II, indicate that there is a significant difference between various methods (inhibitor *versus* no inhibitor) and substrates (hypoxanthine *versus* xanthine). However, there was no significant difference between diets or time intervals. First order interactions were not significant. The unbiased mean titer with inhibitor, 182 μ l. of O_2 per gm. per hour, was significantly greater than that without the inhibitor, 125 μ l. of O_2 per gm. per hour. Although there is no statistically significant difference between intervals, inspection of Table III indicates that there is a larger difference between the first two intervals and the remaining three.

TABLE I

Effect of AHPC on Xanthine Oxidase Titer with Various Substrates (Activity in Microliters of O₂ per Gm. of Liver per Hour)

Hypoxanthine and xanthine, 4×10^{-5} mole per gm. of tissue. AHPC, 1×10^{-5} mole per gm. of tissue.

Rat No.	Diet No.	H - A*	H - E†	X - A‡	X - E§
28	R33	295	250	193	146
29	R33	223	185	166	127
30	R33	118	-48	98	-67
89	R58A	158	134	81	57
91	R58A	216	175	163	125
93	R58A	240	202	228	192

* H - A, difference between O₂ uptake with hypoxanthine and AHPC.

† H - E, difference between O₂ uptake with hypoxanthine and no added substrate.

‡ X - A, difference between O₂ uptake with xanthine and AHPC.

§ X - E, difference between O₂ uptake with xanthine and no added substrate.

TABLE II
Analysis of Variance

Source of variation	Degrees of freedom	Sum of squares of deviations from mean	Variance	Variance ratio
Interval	4	580.5	145.1	
Diet (confounded with rats)	1	120.4	120.4	
Substrate	1	602.1	602.1	4.55*
Inhibitor	1	721.2	721.2	5.45*
First order interactions (total)	15	446.0	29.7	
Error	37	4900.1	132.4	

* Significant at 5 per cent point.

TABLE III

Xanthine Oxidase Activity Determined with and without 2-Amino-4-hydroxy-6-pteridinecarboxaldehyde Activity in Microliters of O₂ per Gm. of Liver

Interval after addition of substrate min.	AHPC absent in endogenous flask	AHPC present in endogenous flask	Mean per interval
20	15.6	53.2	34.4
40	42.0	59.2	50.6
60	47.5	64.0	55.7
80	50.4	63.3	56.9
100	52.3	63.3	57.8
Mean per hr. $\pm$ s.e.	125 $\pm$ 18	182 $\pm$ 18	

DISCUSSION

Kalckar *et al.* (5), Hofstee (6), and Lowry, Bessey, and Crawford (7) have found AHPC to have, even at high dilution, a very strong inhibitory effect on purified xanthine oxidase. Lowry *et al.* have given the dissociation constant (K_i) for this enzyme-inhibitor complex as 6×10^{-10} mole per liter, and the Michaelis-Menten constant (K_m) for xanthine as 9×10^{-7} mole per liter. On the basis of these values, they suggest that xanthine oxidase may be virtually titrated by the use of AHPC. Ackermann and Potter (8), in a recent paper, presented a new theoretical treatment of the relationship of reaction velocity to enzyme concentration in enzyme-inhibitor systems. They show that in the case of a non-competitive inhibitor, *i.e.* when the affinity of the enzyme for the inhibitor is great as compared with that for the substrate, the velocity of the reaction is proportional to a constant times the difference between the total enzyme and total inhibitor concentrations.

$$V = k (E_t - I_t)$$

This relationship may be used to determine the apparent concentration of xanthine oxidase with AHPC as an inhibitor, since the affinity of the enzyme for AHPC is more than 1000 times that for xanthine. Using the above equation, we have determined the apparent velocity constant, k , for xanthine oxidase in rat liver homogenates with AHPC as an inhibitor and hypoxanthine and xanthine as substrates. The values are approximately 10,000 and 5000 respectively. The constant was found to be relatively uniform over a range of 25 to 75 per cent inhibition and to be independent of enzyme concentration.¹ Bain (9), in a study of the kinetics of cholinesterase inhibition, recently confirmed the applicability of the Ackermann-Potter equation given above to irreversible inhibitor-enzyme systems.

It would seem from a consideration of the data presented above that an improved method for the assay of xanthine oxidase may be devised. The use of the inhibitor to free the endogenous respiration of contamination by xanthine oxidase activity results in higher titers and prevents negative results which are obviously artifacts.

We are reasonably sure that the effect of the inhibitor on other enzymes responsible for the endogenous respiration is so small as not to cause an appreciable error in the xanthine oxidase titer. It has been found in this laboratory that the addition of AHPC in proportional quantities does not affect the titers of succinic dehydrogenase and cytochrome oxidase. Lowry and coworkers (7) report that AHPC does not affect the oxidation of reduced diphosphopyridine nucleotide by an enzyme in milk.

¹ Van Meter, J. C., unpublished data.

Examination of Table III shows that the oxygen consumption during the first 40 minutes after addition of substrate is not constant. During the next 60 minutes it is relatively constant. This period conforms to the part of the curve referred to by Axelrod and Elvehjem as linear. It would seem preferable to take a specifically defined time interval and determine the arithmetical mean, rather than to rely on inspection of a curve for determination of activity. In so doing, personal bias, which enters into a calculation based on inspection, is eliminated.

The following recommendations for improvement of the assay of xanthine oxidase in tissue homogenates are made: (a) that 2-amino-4-hydroxy-6-pteridinecarboxaldehyde be used as an inhibitor to remove xanthine oxidase activity from the endogenous respiration; (b) that the xanthine oxidase activity in tissue homogenates be calculated from the mean oxygen consumption due to xanthine oxidase from 40 to 100 minutes after the addition of substrate.

SUMMARY

1. Data are presented on the use of 2-amino-4-hydroxy-6-pteridinecarboxaldehyde in the determination of xanthine oxidase in rat liver homogenates.

2. It is proposed that this compound be used as an inhibitor to remove xanthine oxidase activity from the endogenous respiration and thus increase the accuracy of the method of estimation of xanthine oxidase activity in tissue homogenates.

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A REVISION OF THE SCHOENHEIMER-SPERRY METHOD FOR CHOLESTEROL DETERMINATION

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The original Schoenheimer-Sperry method for cholesterol determination (1) has been modified at several points. It is the purpose of this report to describe the revised procedure with particular emphasis on a recent investigation of the technique used for the precipitation and washing of cholesterol digitonide.

EXPERIMENTAL

Except for changes in the precipitation and washing technique, the modified Schoenheimer-Sperry method was used in the form described below for all determinations of total and free cholesterol. The blood serums studied were obtained from healthy human subjects (2); 1 or 2 ml. were extracted in a total volume of 25 or 50 ml., respectively. Aliquot portions of the extracts were precipitated by four different procedures. In Procedure A, a 0.4 per cent solution of digitonin in water was used with no other changes in the method as described below. This is the procedure which has been employed in our laboratory for some time. The solution was about 2 months old at the time the investigation was started, and about 9 months old at the end. In Procedure B, a dilute alcoholic digitonin solution, prepared as described by Sobel and Mayer (3), was used. This is the modified method, adopted as a result of this study, and described below. Procedure C was the same as Procedure B, except that precipitation was continued for only 3 hours at a temperature of 37°. In Procedure D, free cholesterol was precipitated with the dilute alcoholic digitonin solution for 3 hours at 37°, and stirring rods and the acetone-ether washing were omitted. This is the procedure recommended by Sobel and Mayer (3); it was applied exactly as they described it except that 2 ml. of extract and 1 ml. of digitonin solution were employed instead of the larger volumes used by them. All determinations were carried out in duplicate.

Results

Comparison of Aqueous and Dilute Alcoholic Digitonin Solutions—Procedures A and B were applied to twenty-five samples of serum. In one

sample the total cholesterol values were the same, and in another Procedure A gave a slightly higher result, but in the other twenty-three Procedure B yielded the larger values. In all but one of the free cholesterol determinations Procedure B also gave the higher result. Although many of the differences were small, the consistency of the results leaves no doubt that the alcoholic digitonin solution was superior to the aqueous.

The difference tended to become larger as the aqueous digitonin solution became older; in the first seventeen determinations, carried out when the solution was from 2 to 5 months old, the differences were usually less than 5 per cent. But in all but one of the last eight determinations, when the solution was 7 to 9 months old, the differences ranged from 7 to 12 per cent for total cholesterol, and from 7 to 23 per cent for free cholesterol. Comparisons with standard solutions of cholesterol confirmed the conclusion that the aqueous digitonin solution had degenerated with age.

Effect of Time of Precipitation—Procedure C was applied to eleven samples of serum, and in all cases it gave a lower value than Procedure B for both total and free cholesterol. Although most of the differences were small (1 to 9 per cent), the consistency shows that 3 hours at 37° is not adequate for complete precipitation.

Effect of Omission of Stirring Rods and Acetone-Ether Washing—Procedure D, which was proposed by Sobel and Mayer for free cholesterol determination, was applied to twenty samples of serum, and in all cases it gave a lower result than Procedure B. Although some of the differences were small, in over half they ranged from 5 to 12 per cent. In eight of the eleven instances in which both Procedures C and D were applied to the same serum, the latter gave a lower result. These findings suggest that the use of stirring rods, as described in the original method, is essential to optimal results. The omission of the acetone-ether washing might be expected to increase, rather than decrease, the values obtained for free cholesterol.

Percentage of Free in Total Cholesterol (F:T)—With the exception of slightly high values in Subject 71, all of the percentages of F:T obtained with Procedures A, B, and C were within the normal range of about 24 to 30 per cent (4). Inspection of the data, which are listed in Table I in the order in which they were obtained, suggested that, in the analyses carried out before the aqueous digitonin solution had seriously deteriorated, the variation in F:T was much less among the two or three determinations on a given individual than it was among individuals. Statistical analysis¹ of the data, except the four determinations in December, showed that this impression was correct. By the correlation technique, or by an equivalent analysis of variance, it was found that there was a highly

¹ We are indebted to Dr. John W. Fertig for the statistical analyses.

significant correlation among the values obtained by the different procedures on the same sample of serum.

Error of Method—During the investigation free cholesterol was determined in duplicate portions of serum extract 91 times, and total chole-

TABLE I
Effect of Variation in Method on Percentage of Free in Total Cholesterol

Subject No.	Date of analysis	Free in total cholesterol		
		Procedure A	Procedure B	Procedure C
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	May 24	26.9	27.2	26.7
9	" 31	26.3	26.9	26.0
50	" 31	26.7	26.3	26.2
71	June 7	30.2	30.6	31.1
69	" 10	29.4	29.1	28.3
82	" 21	29.5	30.5	
89	" 23	25.2	24.7	24.8
70	" 23	29.2	27.4	28.5
2	" 27	28.5	29.2	28.9
60	" 28	27.0	26.3	
72	" 28	28.0	28.0	28.2
73	" 28	26.8	27.4	
77	July 5	25.0	26.1	
86	" 13	29.0	29.6	
87	" 27	25.7	26.5	25.9
8	Aug. 15	27.0	27.7	
64	" 15	27.0	26.9	
1	Oct. 5	26.9	26.8	26.9
85	" 17	27.7	27.3	
6	" 21	24.7	25.0	
84	Dec. 12	23.8	25.3	
84	" 27	24.3	27.7	
88	" 29	25.4	27.2	
60	" 29	24.2	28.4	

Each value was calculated from the averages of duplicate determinations of free and of total cholesterol.

terol 70 times. From the differences between the values obtained in pairs of determinations the error of the method was evaluated by statistical treatment¹ of the data. Within the range of concentrations encountered, the error was found to be independent of the concentration. The error also appeared to be independent of the procedure used. Hence, all of the data for each of the cholesterol fractions were treated together to give an error of measurement, represented by a standard deviation of 0.77 mg. per 100 ml. for free cholesterol, and 1.81 mg. per 100 ml. for

total cholesterol. In terms of probable errors the values are, respectively, 0.52 and 1.22 mg. per 100 ml. (*cf.* (4) p. 131).

Precipitation of Cholesterol from Standard Solutions with Different Digitonin Solutions—Aqueous and dilute alcoholic solutions, freshly prepared from seven different lots of digitonin from three manufacturers, precipitated cholesterol quantitatively from two standard solutions in alcohol-acetone. All of the values were between 99 and 101 per cent recovery, except for those of 97 and 98 per cent yielded by one of the aqueous solutions.

Revised Method for Cholesterol Determination

Special Reagents—

1. Solvents. Acetone-absolute ethanol (1:1); acetone-ether (1:2); ether. We make it a practice to use only redistilled solvents and ether which is peroxide-free as tested by shaking 10 ml. with 1 ml. of 10 per cent cadmium potassium iodide solution in a glass-stoppered cylinder. The mixture is placed in a dark place for 1 hour. If any color develops, the ether should be purified by treatment with an acidified 5 per cent solution of ferrous sulfate.

2. Digitonin solution, 0.5 per cent. Dissolve 500 mg. of digitonin in 100 ml. of 50 per cent alcohol (55 ml. of 95 per cent alcohol and 45 ml. of distilled water) at 60° (3).

3. Potassium hydroxide solution made by dissolving 10 gm. of pure KOH in 20 ml. of water. If a sediment develops, the solution is filtered through sintered glass before use.

4. Acetic acid solution made by diluting 10 ml. of glacial acetic acid to 100 ml. with water.

5. Acetic anhydride, 99 to 100 per cent. The Eastman Kodak product has been uniformly satisfactory.

6. Cholesterol solutions, containing exactly 100 mg. per 100 ml., in glacial acetic acid. Working standards are made by suitable dilution of the stock solution with glacial acetic acid. The cholesterol must be anhydrous and pure. It does not keep well under ordinary conditions of storage and should be recrystallized one or more times from an anhydrous solvent and dried thoroughly in a vacuum desiccator over sulfuric acid or phosphorous pentoxide before use in the preparation of standard solutions.

Special Equipment—

1. Funnels, 2.5 cm. in diameter.

2. Filter paper, 4.5 cm. in diameter, extracted with hot alcohol, preferably in a continuous extractor (5), until it is lipide-free. The paper

must be rapid, to minimize evaporation of solvent, and sufficiently retentive. Schleicher and Schüll's Sharkskin meets these requirements.

3. Stirring rods, approximately 13 cm. long.

4. Preserving jars with rubber gaskets.

5. Dropping bottles with ground-in pipettes, equipped with rubber bulbs, for the solvents.

6. Centrifuge tubes, heavy duty, 12 ml., calibrated at 2 ml., and numbered.

7. Dark cabinet, containing a water bath. A wooden packing box, equipped with a door, will serve. We use a box 18 X 10.5 inches, and 9.5 inches high. A copper pan, about 4 inches high just fits in the box. The wooden cover of the pan has thirty holes for centrifuge tubes, and provision is made for a thermometer and a funnel for adding hot or cold water as needed.

8. Photometer. We use the instrument described by Weech (6), but the method may be adapted to other instruments.

Extraction of Blood Serum—About 2 ml. of acetone-ethanol are placed in a 5 ml. volumetric flask, and 0.2 ml. of serum is added slowly in such a manner that it runs down the wall of the flask and forms a layer under the solvent. As soon as the pipette is withdrawn, the contents are mixed thoroughly by a swirling motion. A finely divided precipitate should result. The solvent is brought just to a boil on the steam bath with agitation to prevent bumping, the flask is cooled, acetone-ethanol is added to the mark, and the suspension is thoroughly mixed and filtered into a small test-tube. Aliquots of the clear filtrate for free and total cholesterol determinations are pipetted at once to avoid evaporation.

*Precipitation of Free Cholesterol*²—To 2 ml. of the filtrate in a centrifuge tube 1 drop of the acetic acid solution and 1 ml. of the digitonin solution are added. The contents are stirred thoroughly with a rod which is left in the tube. The tube is placed in a preserving jar which is covered tightly and left overnight at room temperature.

The tube is transferred to a rack, the contents are stirred gently to free precipitate which may adhere to the wall near the surface of the liquid, and the rod is removed without contact with the upper part of the tube and placed on a rack made of heavy wire and so designed that a number of rods may be held without danger of rubbing off adherent precipitate. The position of the rod is noted so that it may be returned to the proper tube. The tube is centrifuged for 15 minutes at about 2800 r.p.m., and

² The procedure is described for a single determination. In practice the average time per analysis may be greatly shortened by carrying through a considerable number of analyses together.

the centrifugate, which should be clear, except for the occasional presence of a few particles floating at the surface, is decanted with a slow, steady motion and watched closely in a good light as the fluid leaves the precipitate. If any is seen to be suspended, the sample is recentrifuged, or better, discarded. If this happens at all frequently, the time or speed of centrifuging should be increased. The tube is drained for a few moments and the last drop is removed by touching the lip to a clean towel. The rod is returned to the tube, the wall of the tube and the rod are washed down with 1.5 to 2 ml. of acetone-ether, the contents are stirred thoroughly, the rod is returned to the rack, the tube is centrifuged for 5 minutes, and the centrifugate is decanted. The precipitate is washed twice more in the same manner with ether. The rod is returned to the tube which may be stored for several days at this stage. If color development is to follow at once, the ether is evaporated by placing the tube for a few minutes in a moderately warm water bath.

Precipitation of Total Cholesterol—1 drop of potassium hydroxide solution is placed in a dry centrifuge tube, 1 ml. of the serum extract is added, and the mixture is stirred with a vigorous up and down motion of a rod until no droplets of the alkali solution can be seen at the tip of the tube. A preserving jar containing a layer of sand about 3 cm. deep is heated in a water bath until the temperature of the sand is 45°. The tube is placed in the sand, and the jar is covered tightly and placed in an incubator at about 38° for 30 minutes.

The tube is removed to a rack and cooled. The rod is raised, acetone-ethanol is added to the 2 ml. mark, and the alkali is neutralized to the phenolphthalein end-point with 10 per cent acetic acid. From 4 to 6 drops should be required if the drop of alkali solution was of proper size. An extra drop of acetic acid and 1 ml. of digitonin solution are added, and the sample is treated as described for free cholesterol, except that the precipitate is washed with ether only once.

Development and Reading of Color—The tubes are placed in order of reading in a sand bath at 110–115° in an oven for 30 minutes. The temperature of the water bath in the dark cabinet is adjusted to 25° and maintained there during the rest of the procedure by the addition of hot or cold water as needed. The sand bath is removed from the oven, and 1 ml. of pure glacial acetic acid is added to the first tube while it is still in the hot sand. The contents are stirred vigorously, and the tube is left in the sand while the acid is being added to the next two or three tubes; 2 or 3 minutes in all. The solution is stirred again, and the tube is removed from the sand, cooled, and placed in the water bath. The process is continued until all the tubes are in the water bath in order of reading. A

tube containing 1 ml. of standard solution (0.1 mg. of cholesterol per 1 ml.) is placed at the beginning, and another at the end of the series.

An amount of acetic anhydride sufficient for the number of samples to be read is placed in a glass-stoppered flask and chilled in an ice bath. With the flask still in the ice bath, concentrated sulfuric acid is added in the proportion of 1 ml. to 20 ml. of acetic anhydride with agitation during the addition. The stopper is inserted, and the flask is removed from the bath, shaken vigorously for a few moments, and returned to the bath. A blank containing 1 ml. of acetic acid and 2 ml. of the reagent is prepared.

About 10 minutes later, when the reagent is thoroughly chilled, the first tube is removed from the 25° water bath and wiped dry, 2 ml. of the cold acetic anhydride-sulfuric acid reagent are added, the contents are stirred vigorously, the rod is removed, and the tube is returned to the bath. If the reading is to be carried out in a photometer which uses matched cuvettes of the test-tube type, the solution is poured at this point into a cuvette, which is placed in the 25° bath. The process is repeated with each tube in the series at time intervals, best controlled by a stop-watch, such that the time of color development before reading is the same for all samples. Although the color is fairly stable at its maximum from 27 to 37 minutes after addition of the reagent, it is best to read all samples after as nearly the same time of color development as possible, preferably 30 to 31 minutes after the acetic anhydride-sulfuric acid solution is added. The temperature of the water bath is noted, and adjusted, if necessary, at frequent intervals during the color development and reading.

The process of reading is not described, since it will vary somewhat with the instrument used. The foregoing procedure yields sufficient solution for the instrument we employ (6), and for some others, but some of the commonly used photometers require a larger volume. The method may be adapted to such instruments by suitable adjustment of the volumes. In general, it may be varied in the following ways to meet special conditions: (1) The proportion of serum volume to extract volume may be decreased to compensate for exceptionally high cholesterol concentrations, but not increased. (2) The volume of extract in which precipitation is carried out may be varied at will, provided that 1 drop of alkali solution per 1 ml. of extract is added in the determination of total cholesterol, and that 1 ml. of digitonin solution is added for each 2 ml. of acetone-ethanol. (3) The volume in which color is developed may be varied without reference to the volume in which precipitation was carried out, provided that 2 ml. of the acetic anhydride-sulfuric acid reagent are added for each 1 ml. of acetic acid.

DISCUSSION

Aqueous solutions of digitonin, necessary under the conditions of the original Schoenheimer-Sperry method, have not been entirely satisfactory, because precipitates form in them on standing to a degree which varies widely among different lots of digitonin from different manufacturers. The present study revealed a far more serious objection to aqueous digitonin solutions. It is evident from the results that such solutions, although usually reliable when freshly prepared, cannot be depended on to keep for long periods of time. This difficulty was not encountered with the Hoffmann-La Roche digitonin, which was used exclusively until the supply was cut off by the war, and we have the strong impression, based on much experience, that aqueous solutions of that product did not deteriorate with age, but it must be confessed that no systematic tests, such as that described here, were carried out. Modifications of the original method, particularly the use of acetic instead of hydrochloric acid in neutralizing the alkali in total cholesterol determinations, have obviated the requirement of an aqueous solution, and the findings of this investigation show that the Sobel-Mayer dilute alcoholic solution is preferable, if used under the conditions described above. It did not give complete precipitation under the conditions proposed by Sobel and Mayer (3).

The finding of a close agreement among the F:T values yielded by Procedures A, B, and C (Table I) is of considerable interest in its bearing on the mechanism of precipitation of cholesterol digitonide. Since the amount of total cholesterol present is about twice that of free cholesterol, whereas the quantity of digitonin and total volume are the same in both samples, the result suggests that the rate of precipitation is independent of the amount of cholesterol, at least within the range of quantities encountered in this study, and that for an unknown reason the rate at which the aqueous digitonin solution, used in Procedure A, was able to precipitate cholesterol was reduced.

The F:T results are also of interest in another connection. The discovery (4) that F:T is maintained within a narrow range of variation in healthy persons has been confirmed by a large number of determinations in this laboratory and by other investigators (7). The proportion is maintained within the same narrow range in all pathological conditions we have studied, except liver disease and acute infection. Because the range of variation is small, little attention has been given to the possible significance of variations among individuals within the range. The statistical analysis of the data of this study shows that the variations in F:T among subjects were real and not due to some chance event. This conclusion was confirmed by a study of the duplicate determinations ob-

tained with Procedure B. Each of the two free cholesterol values was paired at random with one of the total cholesterol concentrations and the F:T values were calculated. These are plotted arbitrarily against each other in Fig. 1. The high correlation, indicated by the chart, was borne out by statistical analysis of the data, and it may be concluded that variations in F:T among individuals within the normal range are to only a small extent the result of experimental error.³ The factors which control F:T in each individual remain to be determined.

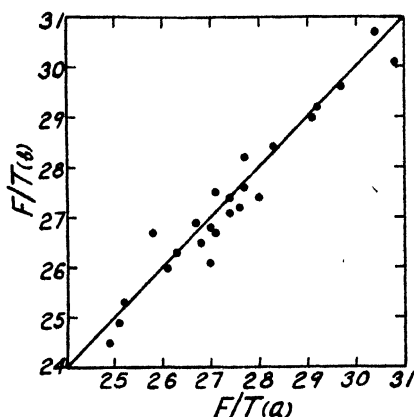


FIG. 1. A comparison of duplicate determinations of the percentage of free in total cholesterol (F:T). One of the duplicates (a), selected at random, is plotted against the other (b).

SUMMARY

A study of the precipitation of cholesterol by digitonin under various conditions within the framework of a revised Schoenheimer-Sperry method gave the following results: (1) Precipitation for 16 to 18 hours at room temperature with solutions of digitonin in approximately 50 per cent alcohol, or with freshly prepared aqueous solutions, yielded maximal values. (2) Aqueous digitonin solutions may lose their ability to precipitate cholesterol quantitatively as they become older. (3) Precipitation is not complete in 3 hours at 37°. (4) The rate of precipitation appears to be independent of the amount of cholesterol present within the range of quantities encountered in this study.

A revision of the Schoenheimer-Sperry method is described.

Variations in the percentage of free in total cholesterol among healthy

³ This conclusion ignores the remote possibility that the extraction of one or the other cholesterol fractions may be incomplete to a variable degree.

persons within the normal range are much larger than can be explained by the error of the determination.

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THE EFFECT OF INCREASING AGE ON SERUM CHOLESTEROL CONCENTRATION

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Determinations of the total cholesterol concentration of the blood serum in twenty-five healthy subjects on two or more occasions over periods of time up to 28 months in length led to the conclusion that the variability of this constituent is much less within a given individual than it is among individuals, and the hypothesis was advanced that in each healthy person the serum cholesterol concentration is maintained, perhaps throughout life, at a constitutional level, from which large deviations do not occur in the absence of unusual stress (1). Other investigators (2-4) have reported findings in general agreement with this hypothesis, and strong support for it was furnished by the finding (5) that the average cholesterol concentrations in children, calculated for each year of age, did not change appreciably from the 2nd month to the 13th year of life and were almost the same as the average found in healthy adults between the ages of 19 and 43 years.

Recently Keys (6) found a considerable increase in the average serum cholesterol concentration of healthy men with increasing age, from 173 mg. per 100 ml. at 19 years to 252 mg. at 52.5 years of age, and Gram and Leverton (7) reported a similar result in women. These findings are inconsistent with the hypothesis stated above.

Because of this apparent discrepancy it seemed worth while to ascertain whether the serum cholesterol level had increased in the individual subjects of the previous study (1) during the 13 to 15 years since that investigation was carried out.

EXPERIMENTAL

During 1949 the cholesterol concentration of the blood serum was determined in twenty-two of the twenty-five subjects of the earlier investigation (1).¹ In two (Subjects 1 and 60) the determination was repeated, and in one (Subject 50) an analysis had been carried out in 1942. The methods are described in the preceding paper (8).

¹ We are deeply indebted to our colleagues who cheerfully furnished samples of their blood; particularly to the ten who participated in the investigation by preparing extracts and mailing them to us.

RESULTS AND DISCUSSION

In interpreting the results (Tables I and II) it is necessary first to consider the possible effect of changes in the Schoenheimer-Sperry method,

TABLE I
Effect of Age on Serum Cholesterol Concentration in Men

Subject No.*	Age in 1949	Average concentration in 1934-36	Results obtained in 1949			
			Procedure A†		Procedure B†	
			Concentration	Change from 1934-36 average	Concentration	Change from 1934-36 average
	yrs.	mg per 100 ml.	mg. per 100 ml.	per cent	mg. per 100 ml.	per cent
8	41	212	191	-9.9	196	-7.5
64	40	251	231	-8.0	236	-6.0
82	53	257	242	-5.8	243	-5.4
85	38	200	176	-12.0	191	-4.5
9	57	255	232	-9.0	245	-3.9
87	45	173	168	-2.9	171	-1.2
2	39	328	329	0.3	338	3.0
89	47	187	192	2.7	201	7.5
84	40	232	237	2.2	266	14.7
1	49	201	226	12.4	235	16.9
1			215	7.0	224	11.4
77	46	201	232	15.4	234	16.4
50	38	315	355	12.7	369	17.1
50		368‡		-3.5§		0.3§
6	51	142	157	10.6	168	18.3
60	32	175	228	30.3	223	27.4
60			208	18.9	230	31.4
Average..		224			237	

* The numbers of the subjects are the same as those used in the previous publication (1).

† Procedures A and B are described in the preceding paper (8).

‡ Analysis in 1942.

§ Change from 1942 value.

|| In calculating this average, an average of the two values for Subjects 1 and 60 was used in each case.

which was used in its original form for the earlier study. Some of the modifications, described in the preceding paper (8), were adopted to facilitate the procedure, and others to minimize the possibility of loss at various points. Thus, if the changes had any effect, it would be in the direction of increasing the values obtained in the present analyses. A more serious difficulty in interpreting the results arises from the finding that

old aqueous digitonin solutions (Procedure A) may give low results as compared with those yielded by a solution in 50 per cent alcohol (Procedure B) (8). It is possible, though unlikely (see the Discussion of the preceding paper), that the earlier determinations suffered from the same source of error, and there is some justification, therefore, for using the aqueous digitonin values (Procedure A) for comparison with the concentrations of cholesterol found about 14 years ago. For this reason the

TABLE II
Effect of Age on Serum Cholesterol Concentration in Women

Subject No.*	Age in 1949	Average concentration in 1934-36	Results obtained in 1949			
			Procedure A*		Procedure B*	
			Concentration	Change from 1934-36 average	Concentration	Change from 1934-36 average
	yrs	mg. per 100 ml.	mg. per 100 ml.	per cent	mg. per 100 ml.	per cent
88	45	202	188	-6.9	213	5.4
86	54	211	225	6.6	232	10.0
70	41	198	223	12.6	244	23.2
72	46	177	207	16.9	219	23.7
71	35	171	215	25.7	220	28.7
83	53	198	227†	14.6	257†	32.1
69	45	162	204	25.9	214	32.1
73	46	182	229	25.8	241	32.4
Average		188			230	

* See Table I.

† This sample of serum had stood for some time at room temperature in a tightly stoppered sterile tube before extraction. The percentage of free in total cholesterol was decreased due to esterification, as would be expected, but since the total cholesterol concentration does not change under these conditions (9), the analysis is included.

values yielded by Procedures A and B are both given in Tables I and II. Actually, the interpretation of the results would not be affected in most of the subjects if the comparison were based on the results of Procedure A.

In the first eight men, as listed in Table I, the cholesterol concentration had not changed appreciably from the average found in 1934-36. Variations of the magnitude found in these subjects have been observed in fasting men during a single morning. In the remaining six men there appear to have been definite increases which were confirmed in two. The average concentration for all of the men was 5.8 per cent higher than the average of the values found in the earlier study. Over a correspond-

ing range of age Keys (6) found an increase in average cholesterol concentration of about 17 per cent, as estimated from a curve plotted from his data.

By contrast to the findings in men, in most of the women there appears to have been a definite increase in serum cholesterol concentration during the period of life studied. The increase, calculated from average concentrations, was 22.7 per cent as compared with an increase, estimated from the data of Gram and Leverton (7), of about 14 per cent over the same life span.

The results lead to two conclusions: The concentration of cholesterol in the blood serum is not maintained by all individuals within as narrow a range as had been thought; in some there is an increase with age, as was indicated by the findings of Keys and of Gram and Leverton. But conversely, the results show that the increase is not an obligatory concomitant of aging, at least over the period of life studied in this investigation.

SUMMARY

The cholesterol concentration of the blood serum was determined in fourteen men and eight women, and the values were compared with those found in the same subjects 13 to 15 years before. In eight of the men and one of the women no appreciable change had occurred, in one woman the result was indecisive, and in six men and six women increases from about 15 to 30 per cent were found. It is concluded that the serum cholesterol concentration increases with age in some persons, but the increase is not an obligatory concomitant of aging.

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THE ENZYMATIC FORMATION OF HYDROXAMIC ACIDS FROM GLUTAMINE AND ASPARAGINE*

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Although glutamine and asparagine have a wide distribution in nature, heretofore the only metabolic function ascribable to them on the basis of known enzymatic processes has been their participation in the metabolism of ammonia (1, 2). We have recently reported the formation of hydroxamic acids by the enzyme-catalyzed replacement of the amide groups of glutamine and asparagine with hydroxylamine (3-5). The same enzyme extract also catalyzes the exchange of the amide group of glutamine and asparagine with (isotopic) ammonia (6). The process would have considerable biological significance if it could be shown that amino acids can participate in an analogous reaction to form peptide bonds. The enzymatic formation of hydroxamic acids from glutamine and asparagine described here is different from the enzymatic synthesis of γ -glutamohydroxamic acid from glutamic acid (7, 8). The latter reaction, which has been used as a model for glutamine synthesis, requires the utilization of adenosinetriphosphate as an external source of energy, whereas the formation of γ -glutamo- and β -aspartohydroxamic acids from the corresponding amides proceeds without the utilization of energy-rich phosphate bonds.

The enzyme preparations which synthesize the hydroxamic acids from the amides and hydroxylamine can also split hydroxamic acids. The enzyme systems are widely distributed in microorganisms.

In the present report the enzymatic synthesis of glutamo- and aspartohydroxamic acids from the two natural amides by enzymes occurring in cell-free extracts of *Proteus vulgaris* X-19 is described in detail. Some observations on the synthesis of glutamine from glutamic acid are also included.

EXPERIMENTAL

For the estimation of the hydroxamic acids, the color reaction with ferric chloride as used by Lipmann and Tuttle (9) was employed. The synthetic hydroxamic acids were prepared to serve as standards.

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β -Aspartohydroxamic Acid (AHA)—This compound was prepared according to the method of Roper and McIlwain from asparagine and hydroxylamine (10). It was recrystallized from ethanol-water mixtures (six times) until the molar extinction with the ferric chloride reaction was constant.

Dibenzoyl- β -aspartohydroxamic Acid—In order to identify the product of the enzymatic exchange, this AHA derivative was prepared by benzoylation in strong alkali. After acidification of the reaction mixture the precipitate was collected, dried, extracted repeatedly with boiling petroleum ether, and recrystallized from ethanol-water and ethanol-ligroin mixtures (m.p. 170°).

$C_{18}H_{16}N_2O_6$ (356). Calculated. C 60.7, H 4.5, N 7.8
Found. " 60.7, " 4.4, " 7.7

γ -Glutamohydroxamic Acid (GHA)—Though some GHA is formed when glutamine is used in a procedure similar to that which yields AHA, it has not been possible to isolate a pure product. However, a satisfactory preparation can be made with γ -ethyl glutamate as the starting material. 25 gm. of hydroxylamine sulfate and 70 gm. of ice were rubbed in a mortar with small amounts of solid barium hydroxide until the pH of the mixture was 8. To 65 ml. of a clear filtrate from this mixture, 8.4 gm. of γ -ethyl glutamate (11) were added. The solution was placed in a desiccator over KOH and was kept at room temperature for 22 hours. The concentrated solution, which had a volume of about 35 ml., was cooled to 0° and was acidified with about 10 ml. of ice-cold concentrated HCl to pH 4. The solution was again placed in the desiccator, which was kept at 2° until the solution had concentrated to a thick, crystalline paste. The crystalline mass was rubbed with 50 ml. of absolute ethanol. The alcohol was decanted and the process repeated. The alcohol-insoluble gum was dissolved in 5 ml. of water and 30 ml. of absolute ethanol were added to it in small portions. The mixture was held at 0–4° overnight. The crystals were filtered, washed with ethanol, suspended in 25 ml. of acetone, and again filtered. The suspension in acetone was repeated and, after filtration, the crystals were washed with ether. The yield of white crystals was 1.6 gm., m.p. 154° (155° (10)). Estimation of the hydroxamic acid grouping by the method of Junnel (12) indicated 99 per cent purity. The crystals were recrystallized (six times) from a water-ethanol mixture until the molar extinction with the ferric chloride test was constant.

Dibenzoyl- γ -glutamohydroxamic Acid—To a chilled solution of 100 mg. of γ -glutamohydroxamic acid were added 500 mg. of magnesium oxide and 600 mg. of benzoyl chloride in three portions. The suspension was kept in an ice bath and shaken vigorously. To the mixture 15 ml. of 10

per cent Na_2CO_3 solution were added and the suspension was filtered. The filtrate was acidified with 5 N H_2SO_4 . The precipitated benzoyl derivative and benzoic acid were filtered, washed with water, and dried. The crystals were washed four times with boiling petroleum ether; the insoluble residue was dissolved in a minimum of hot alcohol, cooled, and excess petroleum ether was added to it. The crystals were collected and recrystallized repeatedly from ethanol-ligroin mixtures. Yield 20 per cent, m.p. 167° .

$\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_4$ (370). Calculated. C 61.6, H 4.9, N 7.6
Found. " 61.3, " 5.0, " 8.0

Preparation of Resting Bacterial Cells—All microorganisms were first grown for 24 hours on nutrient agar in Roux bottles. The bacteria were then washed off the agar with water, the aqueous suspension centrifuged, and the residue washed twice with distilled water. The cells were resuspended in water to a density corresponding to 20 mg. of dry weight per ml. The collection and washing of the bacteria were carried out under aseptic conditions at about 4° .

In order to obtain large quantities of *P. vulgaris* X-19, liquid media were employed. The medium contained per liter Difco Bacto-casamino acid 7.5 gm., ammonium sulfate 0.5 gm., ammonium chloride 0.5 gm., potassium nitrate 1 gm., ferric citrate 3 mg., KH_2PO_4 5 gm., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 50 mg., nicotinamide 1 mg., phenol red 2.5 ml. of a 0.02 per cent solution, and 0.1 ml. of a mixture of equal parts of tributyl citrate and methyl benzoate. The pH was adjusted to 6.8 with 10 N NaOH. Usually 15 liters of medium were employed in one run. The medium was sterilized by autoclaving. A small inoculum of the organisms grown on agar was transferred to the medium, and after 18 hours of incubation at 35° , while growth was still limited, 25 ml. of a sterile solution of 50 per cent glucose were added per liter of medium and a filtered stream of oxygen, containing 5 per cent of CO_2 , was bubbled through the solution. The pH was kept constant by the repeated addition of 10 N NaOH. After 6 to 8 hours of additional incubation the cells were collected on a refrigerated Sharples centrifuge. The yield of dry cells was approximately 2 gm. per liter of medium. Because of the short period of incubation during which active multiplication took place, the crop consisted mainly of young cells. If the organisms were to be used as resting cells, they were washed as described above.

Preparation of Cell-Free Extracts—The harvest of wet cells was lyophilized, and the dry material was shaken with an equal volume of glass beads at a rate of 440 excursions per minute for 1 hour. The powder was suspended in water to yield a 2 or 5 per cent suspension which was dialyzed

for 48 hours against a large volume of distilled water at 4°. The suspension was centrifuged in a refrigerated centrifuge at 13,000 r.p.m. The slightly opalescent supernatant solution served as the source of the enzymes. The total nitrogen content (Kjeldahl) of these extracts was 1 to 1.2 mg. per ml. for a 5 per cent suspension of the original bacterial powder.

Enzymatic Synthesis of GHA or AHA—A typical experiment was carried out as follows: A total volume of 2 ml. containing the amides, hydroxylamine, enzyme extract, and buffer solution (for specific quantities see legends to tables and figures) was incubated at 35°. At the end of the incubation, 1.5 ml. of a solution (9) containing 0.5 ml. of 15 per cent trichloroacetic acid, 0.5 ml. of 2.5 N HCl, and 0.5 ml. of 5 per cent ferric chloride in 0.1 N HCl were added and the reaction mixture centrifuged at 13,000 r.p.m. The optical density of the supernatant solution was determined in a Coleman model 6 spectrophotometer at 500 m μ against a reagent blank. For every experiment blank values were obtained by incubation of solutions of enzyme-hydroxylamine, and amide-hydroxylamine at the appropriate pH. In addition, every enzyme preparation was tested with glutamic acid or aspartic acid and hydroxylamine as substrates in order to exclude the possibility that hydroxamic acids or other chromogenic material might arise by the metabolism of the free dicarboxylic acids.

Ammonia Determinations—These were carried out on an aliquot of a sample deproteinized with an equal volume of 20 per cent trichloroacetic acid. After removal of the precipitate on the centrifuge, 1 ml. of the supernatant served for the determinations, which were carried out in especially designed test-tubes (13) by aeration of the ammonia into boric acid. The protein-free samples were made alkaline by the addition of borate buffer (pH 10) to which sufficient 10 N NaOH had been added to neutralize the trichloroacetic acid. Less than 1 per cent of glutamine and none of the asparagine was decomposed by aeration at pH 10 at room temperature for 30 minutes.

Identification of AHA and GHA Formed by Enzymatic Exchange. *AHA*—To a solution containing 12 mm of hydroxylamine hydrochloride and 6.8 mm of asparagine in a volume of 52.5 ml. at pH 6.2, 7.5 ml. of cell-free *Proteus* extract (1 mg. of N per ml.) were added and the mixture was incubated at 35° for 3.5 hours. The colorimetric estimation indicated the formation of 145 mg. of AHA. 6 ml. of 15 per cent trichloroacetic acid were added to the solution and the precipitate was removed by centrifugation. The supernatant was concentrated to 25 ml. by lyophilization, the pH was adjusted to 4.5, and an equal volume of saturated cupric acetate solution was added to it. After 12 hours in the cold, the copper precipitate was collected, washed with a small volume of water, and decomposed with hydrogen sulfide. The AHA present in the combined filtrate and

wash water from the copper sulfide was benzoylated and purified as described for synthetic AHA. The crude insoluble dibenzoate weighed 240 mg., corresponding to a 70 per cent yield of the amount of AHA estimated by the color reaction. After two recrystallizations from ethanol-water mixtures the melting point was 170° (mixed melting point with synthetic sample 170°).

GHA—A solution containing 4 mm of hydroxylamine hydrochloride and 6.8 mm of glutamine per 100 ml. of water was adjusted to pH 8, and 100 ml. of cell-free *Proteus* extract were added to it. The mixture was incubated at 35° until maximal color development with the ferric chloride test

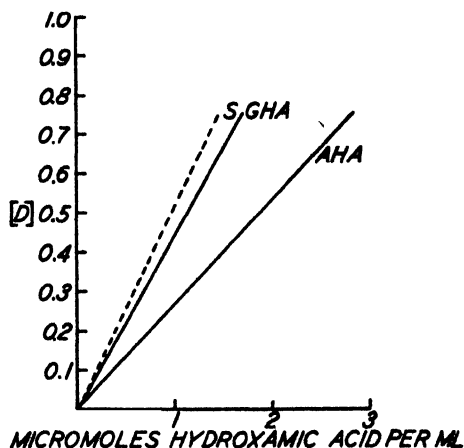


Fig. 1. Optical density of ferric complex of glutamo-, asparto-, and succino-hydroxamic acids. 1.5 ml. of ferric chloride reagent (9) were added to 2 ml. of an aqueous solution of GHA or AHA. The color density was read in No. 6308 cuvettes in the Coleman junior model 6 spectrophotometer at 500 $m\mu$. The optical density of the succino-hydroxamic acid-ferric complex (S) (9) was read at 540 $m\mu$.

was obtained (6 hours). The final determination indicated the formation of 270 mg. of GHA. To the mixture 40 ml. of 15 per cent trichloroacetic acid were added and the precipitate was removed by centrifugation. The copper complex of the hydroxamic acid was obtained and decomposed as described for the asparto analogue and the dibenzoyl derivative was prepared as described for the synthetic GHA. As in the synthetic preparation, the yield of the dibenzoyl derivative was low, owing to the instability of GHA (m.p. 165°, mixed melting point with synthetic sample 165°).

Color Equivalents and Absorption Spectra of AHA and GHA in Ferric Chloride Reaction—The optical density of the ferric complex of AHA is lower than that of GHA, which, in turn, does not quite equal that of the hydroxamic acid from succinic acid anhydride (Fig. 1). The difference

in molar extinction in the ferric chloride reaction with the two homologous hydroxamic acids demonstrates the necessity of basing quantitative estimations of hydroxamic acids on standard curves derived from pure synthetic material.

The absorption maxima of GHA and AHA lie at 500 $m\mu$ (Fig. 2). The absorption curves of solutions containing enzymatically synthesized GHA and AHA coincide with those of the synthetic products over the range

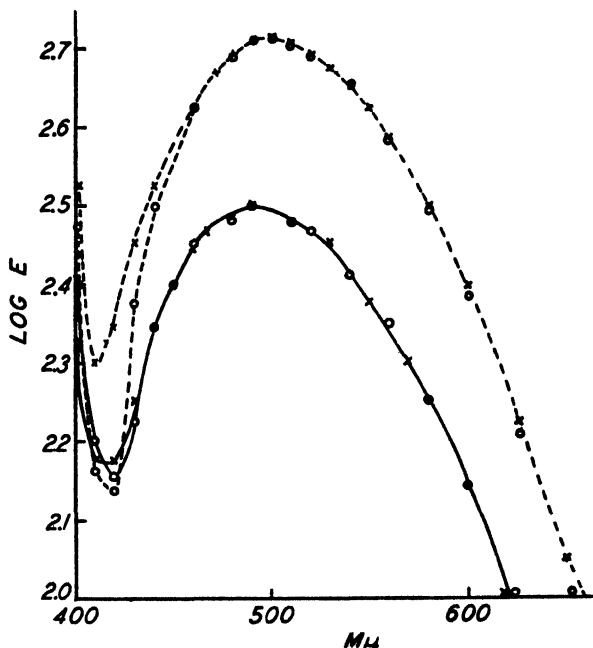


FIG. 2. Absorption curves of ferric complex of synthetic (X) and enzymatically synthesized (O) AHA (solid line) and GHA (broken line). Beckman spectrophotometer.

from 440 to 625 $m\mu$. Below 440 $m\mu$, the spectral absorption of the mixture of ferric chloride and hydroxylamine is different from that of a reagent blank containing only ferric chloride, trichloroacetic acid, and hydrochloric acid. This explains the discrepancy in the absorption curves of synthetic or enzymatically synthesized hydroxamic acids between 400 and 440 $m\mu$.

Dependence of Enzymatic Synthesis of AHA and GHA on pH, Enzyme Concentration, and Time—The pH optimum of the enzymatic synthesis of AHA is at 6.2 and that of GHA at 8.0 (Fig. 3). A considerable splitting of added AHA occurred in the enzyme extract. The pH curve in this case therefore represents the summation of the dependence of the synthesis

and the splitting of AHA on the pH of the solution. This accounts for the second optimum found at about pH 9 to 9.5 at which no splitting of AHA takes place. The pH optimum for the enzymatic hydrolysis of AHA is around pH 7 to 8. On the other hand, the enzyme extracts showed only a weak ability to split added GHA, and, therefore, the curve represents a true picture of the pH dependence of the synthesis of GHA. In all of these experiments not only were controls carried out with enzyme-hydroxylamine and amide-hydroxylamine at the appropriate pH levels, but the influence of the respective buffers on the color development of succino-

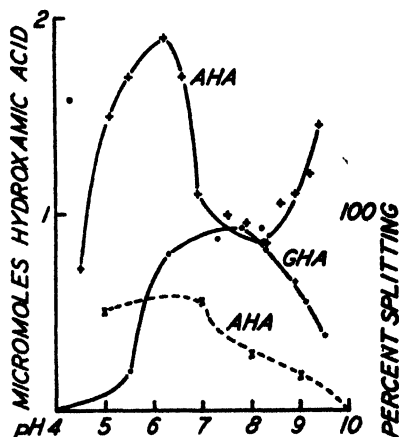


FIG. 3. pH dependence of enzymatic synthesis of GHA and AHA (solid line) and enzymatic splitting of AHA (broken line). AHA (X), GHA (●). Quantities per 2 ml. (in micromoles): asparagine or glutamine 75, hydroxylamine 50, 0.5 ml. of enzyme, buffer up to pH 6.9, 0.05 M acetate, hydroxylamine hydrochloride-NaOH, pH 6.9 to 8.6, 0.04 M veronal, pH 8.6 to 9.4. 1 hour's incubation at 35°. AHA splitting: 1 mM of AHA, 0.1 ml. of enzyme per 2 ml., incubation for 1 hour at 35°.

hydroxamic acid and of AHA and GHA was determined. Sodium acetate-acetic acid, hydroxylamine hydrochloride-NaOH, and veronal-acetic acid mixtures were used as buffers and none of these showed any effect on the color development.

The rate of formation of AHA and GHA in the presence of an excess of substrates was proportional to the enzyme concentration over a considerable range (Fig. 4). The enzymatic synthesis exhibited during the first hours the kinetics of a zero order reaction (Fig. 4).

Activation and Inactivation of Enzymes—The enzymes were inactivated completely when the solution was kept for 10 minutes at 55°. At lower temperatures (between 35–55°), the partial inactivation could be prevented to some extent if one of the amides or hydroxylamine was added prior to

heating. Cysteine, which had no effect on the rate of the synthetic processes, protected the enzymes from inactivation during incubation without substrate (Table I).

Phosphate, magnesium, or manganese, tested over a large range of concentrations, did not affect either the rate or the extent of enzymatic synthesis of AHA or GHA. The synthetic processes seemed to be independent of either oxidative or glycolytic mechanisms, for the syntheses were

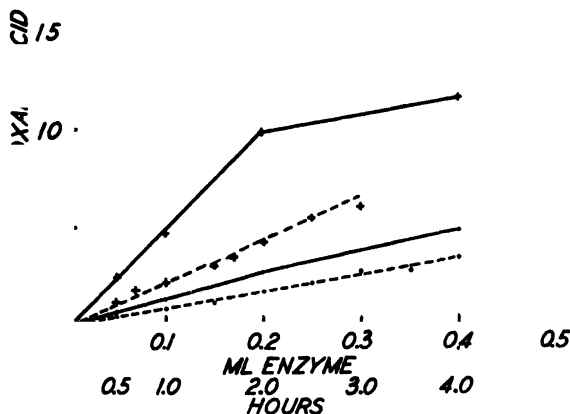


FIG. 4. Dependence of enzymatic synthesis of AHA and GHA on time and enzyme concentration. AHA (×); GHA (●); time dependence, solid lines; enzyme dependence, broken lines. *Time dependence.* For AHA synthesis (quantities per 2 ml.): asparagine 40 μM , hydroxylamine 200 μM , enzyme 0.25 ml., pH 6.2, 35°. For GHA synthesis (quantities per 2 ml.): glutamine 75 μM , hydroxylamine 100 μM , 0.25 ml. of enzyme, pH 8, 35°. *Dependence of enzyme concentration.* For AHA synthesis (quantities per 2 ml.): asparagine 100 μM , hydroxylamine 1000 μM , pH 6.2, 0.5 hour's incubation at 35°. For GHA synthesis (quantities per 2 ml.): glutamine and hydroxylamine 100 μM , pH 8, 1 hour's incubation at 35°; enzyme 1.12 mg. of non-dialyzable N per ml.

unimpaired in the presence of cyanide, fluoride, or of iodoacetic acid (Table II).

Specificity—Acetamide, benzamide, glycynamide, or nicotinamide did not yield hydroxamic acids with enzyme extracts which showed high potency with glutamine or asparagine as substrates.

Ammonia Equivalent (Table III)—In the synthesis of GHA and AHA by exchange of the amide group of asparagine or glutamine with hydroxylamine, the liberation of 1 equivalent of ammonia can be expected. Considerable difficulty was encountered in the experimental verification of the 1:1 ratio of ammonia liberated and of hydroxamic acid formed, probably because of the complexity of the enzymes present in the cell-free extracts.

In addition to its ability to catalyze the synthesis of GHA and AHA, the cell-free extract from *P. vulgaris* possesses a weak ability to split added GHA and a pronounced splitting activity towards AHA. These potencies

TABLE I
Protection of AHA-Synthesizing Enzyme by Substrate and Cysteine

Conditions of preincubation of enzyme (2 hr. period)	Final test	AHA formed after 1 hr. incubation μM
Not preincubated	Complete system	4.2
" "	Without cysteine	4.5
Alone	" "	1.1
Cysteine	Complete system	3.6
Asparagine	Without cysteine	4.5
" + cysteine	Complete system	3.5
Hydroxylamine	Without cysteine	2.9
" + cysteine	Complete system	4.2

The complete system contained 0.25 ml. of enzyme, 10 μM of cysteine, 40 μM of asparagine, and 200 μM of hydroxylamine in 2 ml.; pH 6.2; preincubated and incubated at 35°.

TABLE II
Effect of Cyanide, Fluoride and Iodoacetic Acid on Enzymatic GHA Formation

Substance added	Final concentration	GHA formed μM
	M	μM
Cyanide	0.01	2.8 2.9 3.1
Fluoride-phosphate	0.1, 0.001	2.8* 3.3
Iodoacetic acid	0.01†	3.2

2 ml. contained 50 μM of glutamine, 20 μM of hydroxylamine, and 0.5 ml. of enzyme; incubated for 1 hour at 35°.

* Corrected for depression of color by fluoride and phosphate as measured with synthetic GHA.

† Enzyme preincubated with iodoacetic acid at room temperature for 20 minutes.

may lower the ratio of hydroxamic acid formed to ammonia liberated. Furthermore, the extracts possess weak glutaminase and strong asparaginase activities.

With asparagine as a substrate, all of the amide-ammonia was liberated in the samples with and without addition of hydroxylamine. With glu-

tamine as a substrate, an excess of ammonia was liberated in the presence of hydroxylamine on prolonged incubation. A correction of the ammonia values found in the presence of hydroxylamine by those obtained in its absence, owing to the action of glutaminase, resulted in ammonia values corresponding to less than the expected equivalent to the GHA formed. It appears, therefore, that either glutaminase or asparaginase was the enzyme responsible for the exchange, or that these enzymes were inhibited by the presence of hydroxylamine and the exchange was caused by other enzymes.

TABLE III

Ammonia Liberation During Enzymatic GHA and AHA Formation

Experiment No.	Conditions	Hydroxamic acid formed after					Ammonia formed after				
		1 hr.	1.5 hrs.	2 hrs.	3 hrs.	5 hrs.	1 hr.	1.5 hrs.	2 hrs.	3 hrs.	5 hrs.
		μM	μM	μM	μM	μM	μM	μM	μM	μM	μM
1. GHA	Complete system	5.1		9.7		9.7	4.7*		13.3*		16*
1. "	Without NH_2OH						4		8.6		8.7
2. "	Complete system		6		9	9.3		5.9*		10*	14.5*
2. "	Without NH_2OH							3.2		5.1	7.1
3. AHA	Complete system	5					36*				
3. "	Without NH_2OH						40				

In Experiments 1 and 2, the complete system contained per 2 ml., 0.5 ml. of enzyme, 20 μM of glutamine and hydroxylamine, pH 8, incubated at 35°; Experiment 3, the complete system contained per 2 ml., 0.5 ml. of enzyme, 38 μM of asparagine, and 200 μM of hydroxylamine, pH 6.2; incubated at 35°.

* Corrected for hydroxylamine blank corresponding to 2 per cent of hydroxylamine present.

Occurrence of Enzymes—The enzymes which synthesize and split AHA and GHA are widely distributed among microorganisms (Table IV). In all these experiments the parent amino acids were incubated with hydroxylamine and a cell-free extract or resting cells. These controls were essential in order to exclude the possibility that metabolism of glutamic or aspartic acid may have led to the formation of the corresponding hydroxamic acid. Such controls gave values within the range of blank values obtained on incubation of the enzyme or the amides with hydroxylamine over the whole pH range of GHA and AHA formation above pH 5.5. At pH levels (5.0 to 5.5) below the optimal pH for AHA formation (6.2), solutions of aspartic acid and hydroxylamine with enzyme yielded on incubation considerable color with the ferric chloride reagent. The nature of the chromogenic substance produced under these conditions has not been ascertained.

In a number of the resting cell preparations, the ability to split added synthetic hydroxamic acids was tested. Resting cells of *P. vulgaris* are able to decompose both hydroxamic acids but in the cell-free enzyme extract, prepared as described above, most of the potency towards GHA is lost, whereas that towards AHA is preserved to a large degree. Some microorganisms in the resting state have the ability to synthesize and split GHA and AHA, whereas others are more limited.

Synthesis of GHA and AHA from Corresponding Amino Acids and Their Amides—In the enzymatic synthesis of glutamine from glutamic acid and

TABLE IV
Distribution of Enzymes Synthesizing and Splitting GHA and AHA

	Enzyme preparation	Glutamohydroxamic acid		Aspartohydroxamic acid	
		Synthesis	Splitting	Synthesis	Splitting
<i>Proteus vulgaris</i> X-19.....	Cells	+	+	+	+
" " "	Extract	+	(+)*	+	+
<i>Aerobacter aerogenes</i>	Cells	+	+	—	—
<i>Escherichia coli</i>	"	+		+	
<i>Shigella flexneri</i>	"	—		+	
<i>Pseudomonas aeruginosa</i>	"	—		+	
<i>Staphylococcus aureus</i> ..	"	—		—	
<i>Lactobacillus arabinosus</i>	"	—		—	

In the experiments assaying synthesis with resting cells, 2 ml. contained 20 mg. of dry weight of cells, 75 μ M of glutamine or asparagine, and 200 μ M of hydroxylamine, pH 8 and 6.2 respectively; incubation 1 hour at 35°. In the experiment assaying splitting, 2 ml. contained 20 mg. of dry weight of cells, 1.5 μ M of GHA, or 4.5 μ M of AHA respectively.

* Weak activity.

ammonia, with adenosinetriphosphate as an external source of energy. ammonia may be replaced by hydroxylamine, in which case GHA is formed in place of the amide (7, 8). The cell-free extracts of *P. vulgaris* catalyze the synthesis of glutamine from glutamic acid and ammonia, or of GHA when hydroxylamine is used in lieu of ammonia. In a simultaneous experiment, the formation of GHA from glutamic acid and glutamine in the presence of adenosinetriphosphate and magnesium was determined. The same experiment was carried out with aspartic acid and asparagine as substrates (Table V). Many experiments of this type were conducted and showed the absolute requirement for adenosinetriphosphate and magnesium in the formation of GHA from glutamic acid. On the other hand, neither of these components is needed for the GHA formation from glu-

tamine. In agreement with the observation on the GHA-forming enzyme from pigeon liver (7), no AHA was produced from aspartic acid in a system which synthesized GHA from glutamic acid. The higher blank values found in the experiment with aspartic acid and asparagine are explained by the higher concentration of hydroxylamine and the different batch of cell-free extract used. The effect of adenosinetriphosphate on the formation of hydroxamic acids from glutamine was tested over a large range of concentration (10^{-6} to 10^{-2} M). Whereas an activating effect on the exchange reaction was never observed, high concentrations of the pyrophosphate inhibited the enzymatic formation of GHA from the amide.

TABLE V

Enzymatic GHA and AHA Formation from Glutamic and Aspartic Acids and Amides

	GHA formed from		AHA formed from	
	Glutamic acid	Glutamine	Aspartic acid	Asparagine
	μM	μM	μM	μM
Complete system.	0.5	1.3	0.3	2.1
Without ATP	0.1	1.6	0.3	2.1
“ Mg	0.1		0.3	
“ “ and ATP	0.1	1.6	0.3	2.2
“ amino acids	0.1		0.3	
“ amide		0.1		0.3

The complete system for the experiment with glutamic acid or glutamine contained per 2 ml., 20 μM of amino acid or amide and hydroxylamine, 10 μM of ATP, 2 μM of MgSO_4 , and 0.5 ml. of enzyme, pH 8. For the experiment with aspartic acid or asparagine the system contained per 2 ml., 20 μM of amino acid or amide, 200 μM of hydroxylamine, 10 μM of ATP, 2 μM of MgSO_4 , and 0.3 ml. of enzyme, pH 6.2. Incubation for 1 hour at 35°.

DISCUSSION

In 1889 Hoffmann demonstrated that hydroxamic acids are formed when amides and hydroxylamine are heated in aqueous solution (14). The experiments herein reported show that enzymes occur, widely distributed in nature, which catalyze the exchange of the amide group of the two naturally occurring amides with hydroxylamine. The enzymes occurring in cell-free extracts of *P. vulgaris* are able not only to form glutamohydroxamic acid by exchange of the amide group of glutamine with hydroxylamine but they produce the same compound from glutamic acid and hydroxylamine in the presence of adenosinetriphosphate and magnesium. The fact that no hydroxamic acid whatsoever is formed from glutamic acid without the addition of adenosinetriphosphate and magnesium

indicates that the effective concentrations of both were removed from the cell-free extract, which, without further addition, forms large amounts of hydroxamic acid from glutamine and hydroxylamine. The extract which catalyzes the formation of aspartohydroxamic acid from asparagine and hydroxylamine does not produce the hydroxamic acid from aspartic acid on addition of adenosinetriphosphate and magnesium.

It is not likely that the formation of hydroxamic acids is the primary function of the enzyme system described. It is more probable that hydroxylamine merely substitutes for other amines of biological significance, and thus the naturally occurring amides may be visualized as stores of energy at a level approximating that of a peptide bond. It is now generally assumed that glutamine is enzymatically synthesized through an intermediate acyl phosphate. However, once formed, glutamine may enter into exchange reactions for further syntheses merely by utilization of the energy of the amide bond. Since the mechanism of the exchange may be visualized as the transfer of the glutamyl and aspartyl radicals from ammonia to hydroxylamine or other amines, the terms glutamo- and asparto-transferase activities are suggested for these enzymatic potencies.

A survey of the occurrence of the enzyme system which catalyzes the synthesis or splitting of GHA or AHA showed that the resting cells of some microorganisms possessed all four potencies, while others were more limited. Some were able to synthesize or split only one of the hydroxamic acids. In others none of the activities was found (Table IV). The recently demonstrated exchange of the amide group of glutamine and asparagine with isotopic ammonia (6), catalyzed by the cell-free extracts of *P. vulgaris*, supports the view that the enzymatic exchange with hydroxylamine represents merely a special case of the ability of the amide group of the two naturally occurring amides to serve in exchange reactions with amines. Furthermore, the inability of the resting cells of some microorganisms or of other enzyme preparations to synthesize or split GHA or AHA does not necessarily indicate the absence of enzyme systems which may catalyze the exchange of the amide groups either under different metabolic conditions or with substrates other than hydroxylamine. It is of interest in this connection that resting cells or cell-free extracts of *Streptococcus hemolyticus* (15) or of *Lactobacillus arabinosus*¹ are unable to split glutamine, while actively glycolyzing resting cells of these two organisms can split the amide at a considerable rate. We also found that glycolyzing resting cells of *L. arabinosus* synthesize GHA at a rapid rate from glutamic acid and hydroxylamine, whereas neither resting cells nor cell-free extracts are able to perform the synthesis in the presence of adenosinetriphosphate.¹

On the other hand, the exchanging enzyme system, though present, can-

¹ Owades, P., and Waelsch, H., unpublished.

not always be demonstrated with hydroxylamine as the substrate. Suspensions of rat liver or brain homogenates do not form any hydroxamic acid from glutamine and hydroxylamine, but the exchange of the amide group with isotopic ammonia can be easily shown with such preparations.¹

The general significance of the enzymatically catalyzed exchange reactions in which the amide group of glutamine or asparagine may take part finds support in two recent contributions. An enzyme system was isolated from sugar pumpkin seedlings which catalyzed the exchange of the amide group of glutamine with hydroxylamine or with isotopic ammonia (16, 17). Although this enzyme system also acts by the transfer of the glutamyl radical from ammonia to other amines, it differs from that obtained from microorganisms by its dependence on manganese, phosphate, or arsenate. Some time ago an exchange of the amide group of benzoylglycylamide with aniline, catalyzed by cysteine-activated papain, was demonstrated by Bergmann and Fraenkel-Conrat (18). Recently Fruton has shown that ammonia (isotopic) may replace aniline in the same system (19). Since the free energy change in reactions which involve the exchange of the amide group of naturally occurring or synthetic amides with ammonia or other amines is probably small, the exchange may proceed without the removal of an insoluble reaction product.

SUMMARY

The presence of enzymes which catalyze the exchange of hydroxylamine with the amide group of glutamine and asparagine in cell-free extracts and resting cells of microorganisms is described. The significance of these enzymes lies in their ability to utilize the amide bond energy for further synthetic processes, *e.g.* peptide linkages. The products of the enzymatic exchange, γ -glutamo- and β -aspartohydroxamic acids, were isolated and identified by comparison with the synthetic compounds. The pH optima for the enzymatic synthesis of glutamohydroxamic acid (pH 8) and for aspartohydroxamic acid (pH 6.2) in cell-free extracts of *P. vulgaris* X-19, as well as the stability of the enzymes in the presence and absence of substrate and cysteine, were determined. The presence of phosphate, magnesium, cyanide, iodoacetate, fluoride, or adenosinetriphosphate did not influence the rate or extent of the enzymatic exchange. The cell-free extracts catalyzed the formation of glutamohydroxamic acid from glutamic acid and hydroxylamine but only in the presence of adenosinetriphosphate and magnesium, components which were not needed for the formation of the hydroxamic acid from glutamine. The cell-free enzyme extracts not only catalyzed the replacement of the amide group by hydroxylamine but split added synthetic glutamo- and aspartohydroxamic acids and showed glutaminase and asparaginase activities.

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THE PROTEOLYTIC ACTIVITY OF STRIATED RABBIT MUSCLE*

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The importance of intracellular proteinases in the metabolism of proteins is well recognized. Not only do these enzymes catalyze the hydrolysis of intracellular proteins, but there is reason to believe that they possess the specificities required for protein synthesis (2-4). The rôle of intracellular proteinases of various tissues, normal as well as neoplastic, in autolysis and in exogenous protein hydrolysis has been the subject of numerous studies (5-10). Cathepsins of liver, kidney, and spleen have been characterized with regard to their specificities toward synthetic substrates and have been partially purified (11-13). Cathepsins of other tissues have not been purified beyond the crude extract stage and, with one exception, no clear evidence has been available for the presence of such enzymes in striated muscle tissue. Balls (14), however, described a partially purified cathepsin of beef muscle, possessing a pH optimum of 4.1, which is indifferent to the presence of cyanide or iodoacetate.¹

The present paper describes the isolation of extracts of high proteolytic activity toward hemoglobin and other proteins, prepared from striated rabbit muscle. The enzyme is activated by ferrous ions and its activity is of comparable order of magnitude to that of crystalline chymotrypsin.

EXPERIMENTAL

Measurement of Activity—The procedure used for the determination of proteolytic activity was essentially that of Anson (15). To 5.0 cc. of a urea-denatured hemoglobin solution containing 0.1 M acetate buffer were added 5.0 cc. of the enzyme solution and 5.0 cc. of either distilled water or of a solution containing activators. The resulting reaction mixture was

* Presented before the Forty-first annual meeting of the American Society of Biological Chemists, Atlantic City, April 17-21, 1950 (1).

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¹ The details of this work have come to our attention after completion of the present experiments. It is likely that the proteolytic activity described by Balls is similar to, or identical with, that described here in greater detail.

incubated at 35° at pH 4.0. After a 5 minute equilibration period, 2.0 cc. aliquots were withdrawn at 10 minutes intervals and pipetted into 5.0 cc. of 5 per cent trichloroacetic acid. After standing for 1 hour at room temperature, the trichloroacetate precipitate was centrifuged, and the optical density of the supernatant solution was determined in a Beckman spectrophotometer at 280 m μ . The concentration of enzyme was chosen so that the increase in optical density for the initial 60 minutes was linear with respect to time. Activities were expressed as increase in optical density per hour.

In the presence of ferrous ions, the optical density of the blank (in the absence of enzyme) was not constant but increased with time.² Appropriate corrections were applied in all experiments in which ferrous ions were present.

Results

Preparation of Crude Extract—The rabbit was stunned and killed by severing the cervical arteries. After thoroughly chilling the eviscerated carcass, the muscles of the back and hind legs were dissected and separated as far as possible from fat and connective tissue. The muscles were washed several times with ice water and passed through a previously chilled meat grinder. The ground muscle was dispersed in a Waring blender for 3 minutes with twice the volume of the extracting solution. After standing for 12 hours at 0°, the insoluble residue was removed by centrifugation to yield the crude extract. Various solvents were tested to obtain optimum extraction of proteolytic activity. A summary of the activities obtained with the different extracts is shown in Table I. Although high concentrations of KCl and LiCl yielded extracts with activities higher than that obtained with 2 per cent KCl, these extracts contained considerably more enzymatically inactive protein. For that reason, all subsequent preparations were made with 2 per cent KCl.

Influence of pH on Activity—Fig. 1 illustrates the effect of pH on the proteolytic activity of the crude muscle extract. The optimum pH of 4.0 agrees with the optimum pH found for the autolysis of calf muscle (7). The optimum pH found here is the same as that reported for the proteolytic activity of a glycerol extract obtained from bone marrow (16).

Stability of Activity and Effect of pH on Stability—The effect of pH on the stability of the crude extract was determined by incubating the extract at various pH values for 1 hour at 35°, and then determining the activity at the pH optimum. A summary of these determinations is given in Table II.

² This increase in blank is presumably due to the formation of ferric ion which absorbs light at 280 m μ .

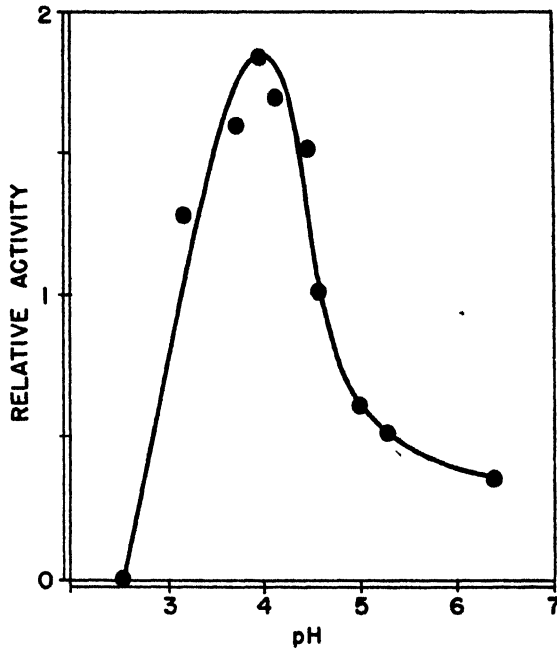


FIG. 1. Effect of pH on the proteolytic activity of the crude muscle extract. All experiments were carried out in 0.033 M acetate buffer at 35°. Activities expressed in arbitrary units.

TABLE I

Proteolytic Activity of Various Types of Muscle Extracts

Ground muscle was extracted with twice the volume of the solvents listed below.

Solvent	Activity*
Water	0.072
2% KCl, pH 6.0	0.169
5% " " 6.0	0.218
1.0 M LiCl, pH 6.0	0.397
50% glycerol	0.028
0.25 M H ₂ SO ₄	0.019
0.15 " acetate buffer, pH 4.5	0.061
0.10 " phosphate buffer, pH 7.5	0.128
0.15 " acetate buffer, pH 4.0, 0.6 M KCl	0.043
0.15 " phosphate buffer, pH 7.5, 0.6 M KCl	0.128

* Activities in arbitrary units (increase in optical densities).

The crude extract may be dialysed overnight at pH 6.0 in the cold with little loss of activity. If the crude extract at pH 6.0 is frozen and

stored at -10° , approximately 75 per cent of the activity remains after 1 week.

Effect of Metallic Ions on Activity—The activating effect of a series of metallic ions was tested on the crude extract. The results are given in Table III. Fig. 2 illustrates the effect of ferrous ion concentration on

TABLE II

pH Stability of Crude Proteolytic Muscle Extract

Samples of the crude extract, obtained with 2 per cent KCl, were incubated for 1 hour at 35° in solutions of varying pH, as given in the first column. The activity was determined in the usual manner and is given in the second column as per cent of the original activity. The extract contained 2.0 mg. of protein N per cc.

pH of incubation	Activity remaining per cent
3.1	46
4.1	69
5.0	65
6.0	85
7.0	46
8.0	37

TABLE III

Influence of Metallic Ions on Proteolytic Activity of Crude Muscle Extracts

The experiments were performed with a crude extract of rabbit muscle which had been partially purified by precipitation of inert protein at pH 5.0, 35° . The enzyme solution contained 1.05 mg. of protein N per cc.

Activator	Activity*
None	0.169
0.01 M barium acetate.	0.134
0.01 " zinc acetate.	0.162
0.01 " ferrous ammonium sulfate.	0.500
0.01 " magnesium sulfate.	0.245
0.01 " manganese "	0.293
0.02 " cobalt sulfate	0.185
0.02 " calcium chloride	0.163

* Activity expressed in arbitrary units (increase in optical densities).

the activity. The activation by ferrous ions is similar when purified preparations of the enzyme are used. However, maximum activation is obtained at lower concentrations of ferrous ions (0.02 M). Cysteine, which produces no change in the activity when added to the extract, completely abolishes the activation by ferrous ion, presumably by complex formation.

Relation of Time and Enzyme Concentration to Extent of Hydrolysis—

The rate of enzymatic hydrolysis at low enzyme concentrations is linear with respect to time and proportional to the enzyme concentration. This is shown in Fig. 3, in which the results are presented for four experiments in which the enzyme concentration was varied over a 4-fold range. At each enzyme concentration, the increase in optical density, which is proportional to the amount of hydrolysis, is practically linear with respect to time. In the inset graph, in which the velocity is plotted against enzyme concentration, is shown the proportionality between velocity and enzyme concentration.

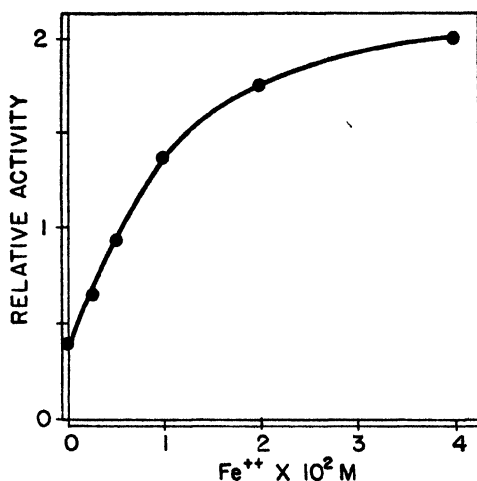


FIG. 2. Effect of ferrous ions on the activity of the crude muscle extract at 35°. All experiments were carried out in 0.033 M acetate buffer, pH 4.0. Activities expressed in arbitrary units.

Purification of Activity—A procedure has been developed in which the specific activity, as measured by hemoglobin hydrolysis, has been increased 1100-fold. Ground rabbit muscle was extracted, as already described, to yield the crude extract. The pH of the extract was approximately 6.0. The pH was adjusted to 5.0 by the addition of 1 N HCl and the extract was placed in a water bath at 35° for 10 minutes. During this time approximately 50 per cent of the protein precipitated. This precipitate was removed by centrifugation. The supernatant solution, containing about 85 to 120 per cent of the original activity, was cooled to 0°, and sufficient 10 per cent sodium trichloroacetate was added to produce a 1 per cent solution of this salt. The pH was adjusted to 4.0 by the dropwise addition of 1 N HCl. The precipitate was removed by centrifugation at 0°, and the supernatant, after being adjusted to pH 6.0, was dialyzed over-

night at 4° in a rapid equilibrium dialyzer^a against 2 per cent KCl. This dialyzed solution was made 0.02 M with respect to zinc acetate and adjusted to pH 5.8. The active protein was precipitated as the zinc salt in 19 per cent ethanol (17) by the following procedure. Ethanol was added slowly

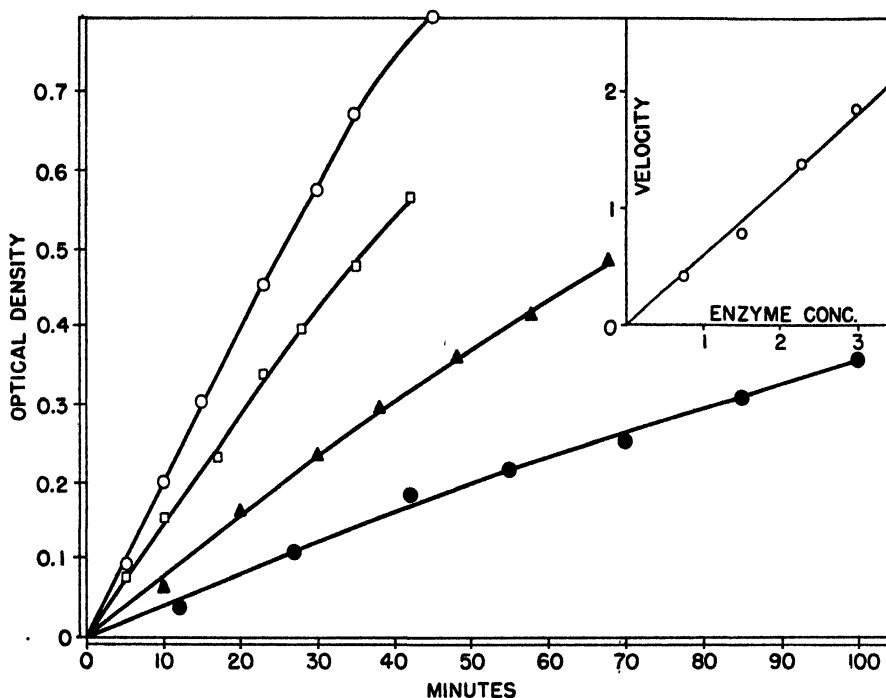


FIG. 3. Hydrolysis of hemoglobin by various concentrations of muscle extract at 35°, pH 4.0, and in the presence of 0.04 M ferrous ions. The muscle extract used was partially purified by the precipitation of inert protein at pH 5.0, 35°. The concentration of muscle protein N per cc. of reaction mixture is indicated as follows: ○ 0.75 mg., □ 0.57, ▲ 0.38, ● 0.19. The inset graph indicates the relation between velocity and enzyme concentration. The velocity is expressed as increase in optical density per minute $\times 10^{-2}$. The enzyme concentrations, which in the inset graph are plotted on a relative scale, are the same concentrations as those indicated for the main graph.

with stirring until the concentration of alcohol was 19 per cent by volume. During the addition of alcohol, the temperature was lowered from 0° to -8°. The precipitate (Precipitate A, Table IV) was removed by centrifugation at -8°. The supernatant was then adjusted to pH 7.5 by the slow addition of 1 N NaOH at -8°. The precipitate (Precipitate B, Table

^a Designed by Dr. W. F. H. M. Mommaerts of this laboratory.

IV) was separated in the same manner as was Precipitate A. Both zinc salt precipitates were dissolved in 0.02 M citrate buffer. The solutions were adjusted to pH 6.0 and dialyzed overnight at 4° against 2 per cent KCl. A summary of a typical purification process is given in Table IV.

Use of Other Substrates—In addition to hemoglobin, the muscle enzyme catalyzes the hydrolysis of serum albumin and casein, although at a slower rate. The muscle enzyme failed to hydrolyze benzoyl-L-arginine ethyl ester, acetyl-L-tyrosine ethyl ester, and carbobenzoxy-L-glutamyl-L-tyrosine,⁴ the specific substrates of trypsin (18), chymotrypsin (19), and pepsin (20), respectively.

TABLE IV
Purification of Proteolytic Activity of Muscle

The starting material was 185 gm. of ground rabbit muscle which was extracted with 370 cc. of 2 per cent KCl. All activity measurements were made in the presence of 0.04 M Fe⁺⁺.

Step	Volume	Protein N	Total activity*	Specific activity†
	cc.	mg. per cc.		
2% KCl extract	220	2.0	12.0	0.40
Treatment at 35°, pH 5.0	204	1.2	13.9	0.84
Trichloroacetate precipitation	222	0.2	10.9	3.60
Zn salt ppt.				
Ppt. A, obtained at pH 5.8	57.5	0.57	209	95.5
" B, 7.5	59.0	0.10	176	447.0

* In arbitrary units.

† Specific activity is expressed as increase in optical density per hour per mg. of muscle protein nitrogen in 1 cc. of reaction mixture.

DISCUSSION

Previous work indicated that the proteolytic activity of striated muscle, measured either by autolysis or by hydrolysis of other protein substrates, is considerably lower than that of soft tissues such as liver, spleen, and kidney (5-9, 21). More recently, it has been shown that the rate of protein turnover in muscle is extremely slow compared to the rate of turnover in the vital organs (22). It was to be anticipated, therefore, that the intracellular proteinases of muscle would either be present in low concentration or else in moderate concentration but of low specific activity.

If the specific activity of the most highly purified muscle extract, measured at pH 4.0 in the presence of ferrous ions, toward hemoglobin substrate is compared to that of crystalline chymotrypsin toward the same substrate

⁴ We are indebted to Dr. J. S. Fruton for the supply of this substrate.

at pH 7.8, it is found that the highest proteolytic activity of the muscle extract is about one-third of that of chymotrypsin (447 compared to 1380). The total activity which could be obtained from 185 gm. of wet rabbit muscle is equivalent in activity to 26 mg. of crystalline chymotrypsin. It should be noted, however, that the total activity (the sum of the activities of the two zinc salt precipitates) has increased 32-fold during the purification process and that this increase has been brought about almost exclusively during the zinc salt precipitation. While the reason for this activation is as yet unknown, two possible mechanisms may be suggested: (1) an inactive precursor is converted to the active form, or (2) an enzyme inhibitor is removed by zinc salt precipitation.

The present findings suggest that striated muscle is a relatively rich potential source of an enzyme of high proteolytic activity and that the low activity, previously inferred from studies on autolysis and turnover rates, is the result of an inhibiting mechanism which controls the intracellular hydrolysis-synthesis equilibrium.

The present proteolytic enzyme shares with some intracellular proteolytic enzymes a relatively low pH optimum, and, with others, the activation by ferrous ions. Thus Stern (7) reported the activating effect of manganese, zinc, and ferrous ions on the autolysis of liver, spleen, and kidney. Activation of leucylglycine dipeptidase by ferrous ions was recently demonstrated (23). Unlike most cathepsins, however, the muscle proteinase described herein is not activated by cysteine.

It is apparent that the present studies which have led to the finding of a highly active source of proteolytic activity of striated muscle will have to be extended to studies on the further purification of the enzyme, on its physicochemical characterization, and on the determination of substrate specificity toward synthetic peptide derivatives. Such studies are now in progress.

This work has been supported in part by the United States Public Health Service, National Institutes of Health.

SUMMARY

From ground, striated rabbit muscle, extracts have been prepared which exhibit proteolytic activity when tested against denatured hemoglobin, casein, and serum albumin as substrates. The proteolytic activity has a pH optimum at pH 4.0 and maximum stability at pH 6.0.

Ferrous ions (0.04 M) cause a 5-fold increase in activity; magnesium and manganese ions are less effective. Other ions that have been tested, including cysteine, produce no activation. The rate of hemoglobin hydro-

lysis is linear with respect to time and proportional to the amount of extract used.

Partial purification of the muscle enzyme has been achieved by a procedure involving partial precipitation of inactive protein at 35°, fractional precipitation with trichloroacetate at pH 4.0, and precipitation of the active protein as the zinc salt in 19 per cent ethanol. Active fractions thus prepared were, per mg. of nitrogen, one-third as active at pH 4.0 as was crystalline chymotrypsin at pH 7.8. The total activity obtained from 185 gm. of wet muscle was equivalent to 26 mg. of crystalline chymotrypsin.

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STEROID EXCRETION IN A CASE OF ADRENOCORTICAL CARCINOMA

V. Δ^4 -PREGNENETRIOL- 3β , 17α , 20α *

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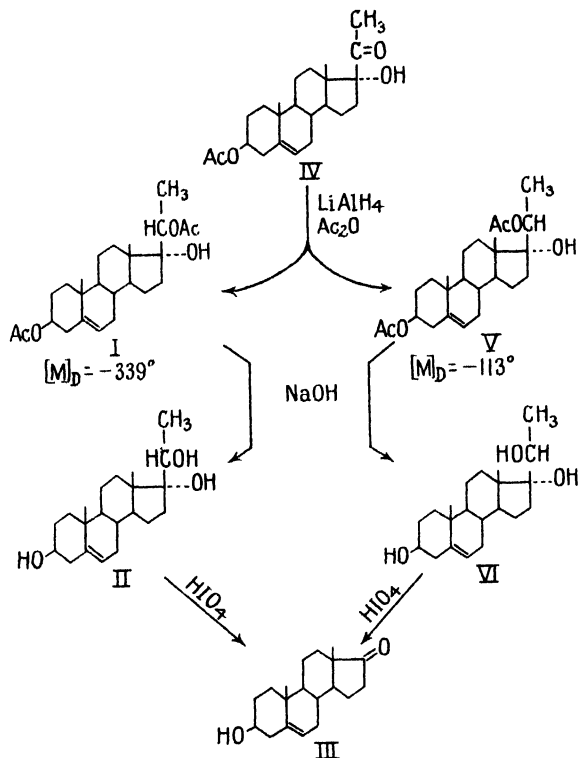
The examination of the 3β fraction of non-ketonic compounds from the urine of a boy with an adrenocortical tumor revealed the presence of three substances which appeared to be different from any steroids hitherto described (1). One of these has recently been identified as Δ^4 -pregnenetriol- 3β , 16α , 20α (2).¹ Since then 13 mg. of a second of these substances (Compound C) were accumulated which allowed the elucidation of its structure as Δ^4 -pregnenetriol- 3β , 17α , 20α .

The reported analysis of the acetate of Compound C (I) was considered to be indicative of a diacetate of a compound $C_{20}H_{32}O_3 \pm CH_2$ (1). Further analysis of this product has given values in excellent agreement with the composition of the higher homologue ($C_{25}H_{40}O_3$). The dried parent compound proved to be hygroscopic and after exposure to the atmosphere analyzed for the hemihydrate of $C_{21}H_{34}O_3$. It seemed probable, therefore, that the analytical data obtained for the dried preparation which are low in carbon reflected a residuum of water rather than a composition different from $C_{21}H_{34}O_3$ for the anhydrous substance. Final and convincing proof for the correctness of the C_{21} formula came from the synthesis of Compound C which is reported below. Examination of the infra-red spectrum of the diacetate showed an absorption peak at 2.78μ , which is characteristic of an O—H bond. It was concluded that Compound C was a triol with one inert and two reactive hydroxyl groups. This in turn suggested the presence of one olefinic double bond, if the compound

* This investigation has been supported by grants from the Bourne Fund and from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

¹ The stereochemical nomenclature employed conforms to current usage, which frequently differs from that in the references cited. Details concerning the use of the terms α and β for most of the relevant positions were specified in the preceding paper (2). In addition, it should be noted that 3-chlorosteroids are now designated in conformity with Shoppee (3), and 17α -hydroxy-D-homosteroids are renamed in view of the evidence presented by Goldberg *et al.* (4). The 20-hydroxysteroids of the 17-iso series which have not been related with certainty to those of the normal series (5) are designated " α " and " β " in accordance with Fieser and Fieser (6).

$C_{21}H_{34}O_3$ is tetracyclic. The most likely locations of an unreactive hydroxyl group in a urinary steroid are the 11β and 17α positions. In general substitutions at these sites have a definite effect on the optical rotation of the molecule and can be distinguished by the sign of their contribution. Since compound I was found to be strongly levorotatory ($[M]_D = -331^\circ$), the presence of the dextro-displacing 11β -hydroxyl group (7) was considered to be unlikely. Moreover, the molecular rota-



tion of the isolated diacetate differs from that of allopregnanetriol- 3β , 17α , 20α diacetate (8, 9) by 203° , a value compatible with the shift observed for reduction of a 5-6 double bond if measurements are made in alcohol or acetone (10, 2).² It seemed possible, therefore, that the isolated com-

² In discussing the correlation between location of the double bond and the shift of rotation on reduction (2) we unfortunately were unaware of the recent simultaneous publications of Barton and Rosenfelder (11) and of Wintersteiner and Moore (12) which give satisfactory reference data for the 6-7 double bond. The values are significantly different from those observed on reduction of the 5-6 double bond. ($\Delta[M]_D^{acet.} - \Delta^\delta = 441^\circ$ for Δ^δ -cholestenol- 3β acetate in chloroform.)

pound was Δ^5 -pregnenetriol-3 β ,17 α ,20 α . In order to test this hypothesis, the triol (II) was treated with periodic acid. The reaction product (III) in carbon disulfide showed an absorption maximum at 5.74 μ , as has been observed with numerous 17-ketosteroids (13). The absorption in the finger-print region of infra-red radiation was in very close agreement with that given by a reference sample of dehydroisoandrosterone. Moreover, benzylation yielded a sparingly soluble derivative that showed no depression of its melting point when mixed with authentic dehydroisoandrosterone benzoate. The identification of the oxidation product (III) as dehydroisoandrosterone establishes in the starting compound (II) the nature and configuration of the ring system, the location of the double bond, the locations of the three hydroxyl groups, and the β configuration of the substituent at C-3, but leaves the spatial arrangement of the hydroxyl groups at C-17 and C-20 undetermined.

While the optical rotation of the diacetate agrees well with the value calculated for the 17 α ,20 α configuration, it does not differ sufficiently from the expected value of 17-iso- Δ^5 -pregnenetriol-3 β ,17 β ,20" β " diacetate³ to permit the elimination of this structure with assurance. In order to prove the configuration of the isolated compound, it was related through synthesis to a starting compound with known orientation of the 17-hydroxy group, Δ^5 -pregnenediol-3 β ,17 α -one-20. This substance was available through isolation from the ketonic fraction of the same urine (18). Its configuration at C-17 has been determined by Reichstein and his collaborators (19, 20) who obtained it by synthesis. The 3-monoacetate (IV) of this compound was reduced with lithium aluminum hydride. This method when applied to other 20-ketosteroids (9, 2) has given a mixture of both 20-epimeric alcohols in which the β isomer appeared to predominate. In this case again two products were obtained which were separated after acetylation. The isomeric diacetates (I and V) were converted to the free triols (II and VI) which were degraded with periodic

³ A product to which this structure has been assigned has been described by Butenandt *et al.* (14). Recent experiments of Salamon (15), however, strongly indicate that this material is a molecular compound of the diacetates of Δ^4 -pregnenetriol-3 β ,17 α ,20 β and of a structural isomer, possibly Δ^5 -pregnenetriol-3 β ,16 α ,17 α . These findings would explain the conversion of Butenandt's product to 17-iso- Δ^5 -pregnenol-3 β -one-20 acetate on heating with zinc (14), if it is assumed that the 3,20-diacetate in the mixture is the actual starting material (16, 17). It is less apparent, however, how Salamon's results are to be reconciled with the observations of Fieser and Huang-Minlon (17) who obtained Δ^5 -pregnenol-3 β -one-20 acetate on repeating Butenandt's experiments. In spite of this uncertainty, it seems warranted to consider Salamon's new diacetate (melting at 185°) (15) as authentic 17-isoallopregnanetriol-3 β ,17 β ,20" β " diacetate and to use its molecular rotation (-67° in acetone) to estimate that of the diacetate of 17-iso- Δ^5 -pregnenetriol-3 β ,17 β ,20" β " (about -286° in alcohol or acetone).

acid. In both series the reaction product was dehydroisoandrosterone (III). It follows that both triols are formed without rearrangement to Δ^5 -homosteroids and therefore represent the two diastereoisomers of Δ^5 -pregnenetriol- $3\beta,17\alpha,20$. The physical constants of the lower melting diacetate (V) and of its parent compound leave little doubt that this triol is the isomer identical with the Δ^5 -pregnenetriol- $3\beta,17\alpha,20\beta$ synthesized by Butenandt, Schmidt-Thomé, and Paul (14). Since its diacetate has been reduced (21) to the diacetate of Reichstein's Compound J (allopregnanetriol- $3\beta,17\alpha,20\beta$), the reference compound for the 20β configuration in 17-hydroxysteroids (6), it follows that our higher melting reaction product, the diacetate I, must have the 20α orientation. It is of interest

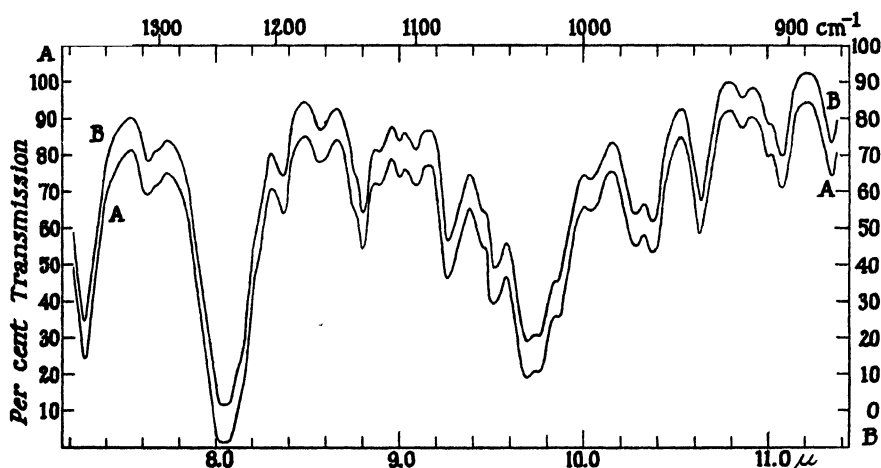


FIG. 1. Infra-red spectrum of Δ^5 -pregnenetriol- $3\beta,17\alpha,20\alpha$ 3,20-diacetate (I). Curve A (synthetic preparation) is drawn with reference to the left ordinate, Curve B (isolated) to that at the right.

that it was obtained as the main product of the reduction. This compound proved to be identical with the isolated substance, as was shown by a comparison of the melting points, of the rotations, and most convincingly of their infra-red spectra (Fig. 1). The free triols, like the diacetates, showed no depression of their melting points on admixture. This identity fully confirms the results of the degradation of Compound C and establishes its structure as Δ^5 -pregnenetriol- $3\beta,17\alpha,20\alpha$.

These findings complete the identification of seven substances isolated from this tumor urine which were not previously known to occur in human urine. Only one of these, Δ^5 -androstenetriol- $3\beta,16\alpha,17\beta$ (22), has since been reported to occur in low concentrations in normal urine as well (23), but the excretion of small amounts of the other substances (with the exception of 17α -methyl- Δ^5 - Δ -homoandrostenediol- $3\beta,17\alpha\beta$ -one-17 which

most probably is an artifact (18)) by normal men and women is considered not only possible but even probable. We have felt justified, therefore, in considering the adrenal tumor as the most probable site of origin of these compounds only if their urinary titers greatly exceed their (for the most part as yet hypothetical) normal levels. The compounds described thus far were obtained in amounts so large that their presence in similar concentration in normal urine is highly improbable. Although the average yield of Δ^5 -pregnenetriol- $3\beta,17\alpha,20\alpha$ (1.3 mg. per liter of urine) was considerably lower, we do not doubt its adrenal origin, since the same urine had yielded much larger amounts of the closely related Δ^5 -pregnenediol- $3\beta,17\alpha$ -one-20 (18). Since several 20-ketosteroids that were administered to man were shown (24-27) to be converted to 20α -hydroxy-steroids, a metabolic interrelationship between Δ^5 -pregnenediol- $3\beta,17\alpha$ -one-20 and Δ^5 -pregnenetriol- $3\beta,17\alpha,20\alpha$ seems a very likely explanation of the presence of both compounds in the same urine extract.

Δ^5 -Pregnenetriol- $3\beta,17\alpha,20\alpha$ agrees with the other compounds⁴ reported for this case in the presence of the 5-6 double bond and in the β configuration of the substituent at C-3. The examination of the acetylated non-ketonic material that failed to precipitate with digitonin has revealed little which would alter the view that the Δ^5 - 3β -stenols represent the predominating type of steroid excretion products in this case. The bulk of the crystalline material obtained from this fraction consisted of the diacetate of Δ^5 -androstenediol- $3\beta,17\beta$ (which forms a rather soluble digitonide). We also obtained small amounts of pregnenediol- $3\alpha,20\alpha$ diacetate (1.5 mg. of diol per liter of urine) and of four unidentified compounds. One of these, the acetate of a benzenoid trienol, has been described briefly (28); the others were obtained in traces and have been characterized only by their melting points (131-133.5°, 209.5-210.5°, and 226-228°, respectively). It would appear from the data obtained thus far that the shift in excretion products from the normally predominant 3α -stanols to the Δ^5 - 3β -stenols is more general in this adrenal tumor case than in any other of which we are aware. Hypotheses have been set forth (1, 29) to explain this shift, but as yet none has received sufficient experimental support to permit its adoption (29).

EXPERIMENTAL⁵

Isolated Δ^5 -Pregnenetriol- $3\beta,17\alpha,20\alpha$ 3,20-Diacetate (I)—The isolation of the diacetate (Compound C acetate) from the remaining extracts (22)

⁴ Δ^5 -Androstetriol- $3\beta,16\alpha,17\beta$ (22), Δ^5 -androstenediol- $3\beta,17\beta$ (1), Δ^5 -pregnenediol- $3\beta,20\alpha$ (1), Δ^5 -pregnenediol- $3\beta,17\alpha$ -one-20 (18), 17 α -methyl- Δ^5 - Δ -homoandrostenediol- $3\beta,17\alpha\beta$ -one-17 (18), Δ^5 -pregnenetriol- $3\beta,16\alpha,20\alpha$ (2), dehydroisandrosterone (18), 3β -chloro- Δ^5 -androstene-17 (18).

⁵ All melting points reported are corrected. Compounds were dried for analysis and rotation at 110° unless noted otherwise. The rotations were measured in 95

of the patient's urine followed the procedure described (1) except that our standard size columns ($r^2h/w = 10 \text{ cm.}^2$ per gm., where r = radius, h = height of the column, and w = weight of material) were found to be preferable for rechromatographing the crude diacetate from alumina to the shorter ones used before. The final product melted at $203\text{--}206^\circ$.

<i>Analysis</i> — $\text{C}_{24}\text{H}_{36}\text{O}_5$.	Calculated.	C 71.25, H 8.97
$\text{C}_{24}\text{H}_{36}\text{O}_5$.	"	" 71.74, " 9.15
	Found.	" 71.76, " 9.18
<i>Rotation</i> — $[\alpha]_D^{25} = -79^\circ$ ($c = 0.38$)		

The main absorption maxima in the accessible finger-print region of infra-red radiation (Fig. 1, Curve B) were at 13.87 , 12.50 , 12.32 , 11.345 , 11.07 , 10.86 , 10.63 , 10.37 , 10.28 , 10.05 , 9.69 , 9.51 , 9.26 , 9.09 , 9.00 , 8.80 , 8.57 , 8.37 , 7.63 , and 7.28μ . The ester peaks were at 8.02 (broad) and 5.74μ , and the hydroxyl peak at 2.78μ . The material studied was an aliquot of the product analyzed and was dried under the same conditions. The hydroxyl peak, therefore, cannot be ascribed to solvent retention.

Isolated Δ^5 -Pregnenetriol- 3β , 17α , 20α (II)—The isolated diacetate (I) was hydrolyzed as described before (1). The triol was recrystallized from 95 per cent alcohol and from acetone and melted with some browning at $222\text{--}225^\circ$. A sample was dried at 110° *in vacuo* for 3 hours and sent to Dr. Huffman for analysis. On redrying at 100° *in vacuo* for 1 hour it lost 2.10 per cent of its weight but regained it rapidly on exposure to the atmosphere (most of it within 5 minutes). The rehydrated product was analyzed. The first set of figures (a) is based on the weight of the sample before redrying, the second (b) on that of the dried sample and on a water weight which is corrected for the weight increase on exposure to air.

<i>Analysis</i> —(a) $\text{C}_{20}\text{H}_{32}\text{O}_3 \cdot \frac{1}{2}\text{H}_2\text{O}$.	Calculated.	C 72.90, H 10.10
$\text{C}_{20}\text{H}_{34}\text{O}_3 \cdot \frac{1}{2}\text{H}_2\text{O}$.	"	" 73.43, " 10.27
	Found.	" 73.34, " 10.32
(b) $\text{C}_{20}\text{H}_{32}\text{O}_3$.	Calculated.	C 74.96, H 10.07
$\text{C}_{20}\text{H}_{34}\text{O}_3$.	"	" 75.40, " 10.25
	Found.	" 74.91, " 10.29

Lack of material prevented the analysis of a more rigorously dried specimen. Since the hydrogen percentage in contrast to the carbon percentage of the compound is essentially independent of the state of hydration, it is under the circumstances probably a more reliable indicator of the composition of the anhydrous steroid.

Degradation of Isolated Δ^5 -Pregnenetriol- 3β , 17α , 20α (II)—2.6 mg. of

per cent ethanol, the infra-red spectra in 1 per cent solutions in carbon disulfide. The spectrometer and cell were as previously described (2) except that the resolution has been raised to its maximal value.

compound II in 1.2 cc. of methanol were treated with 0.15 cc. of a 5 per cent aqueous solution of periodic acid. After the mixture had stood at room temperature for 18 hours, it was distributed between ether and water. The ether layer was washed with a sodium carbonate solution and with water and evaporated. A solution of the residue in carbon disulfide showed no significant difference in its infra-red transmission spectrum from that of dehydroisoandrosterone. Treatment of a pyridine solution (1 cc.) of the oxidation product with 0.04 cc. of benzoyl chloride for 17 hours at room temperature yielded 3.4 mg. of benzoate which was recrystallized three times from acetone. It melted at 254–258° with decomposition and at 254.5–258.5° when mixed with authentic dehydroisoandrosterone benzoate melting at 254.5–257°.

Reduction of Δ^5 -Pregnenediol-3 β ,17 α -one-20 Acetate (IV)—Compound IV was isolated from the tumor urine extracts by the old procedure (18) except that a 1:1 mixture of magnesium silicate and celite (30) was substituted for alumina in chromatographing. A solution of 31.9 mg. of acetate IV in 25 cc. of dry ether was added to a stirred solution of 67 mg. of lithium aluminum hydride in 3 cc. of ether. Stirring under anhydrous conditions was continued for 30 minutes, when the excess reagent was decomposed by the addition of an ether-alcohol mixture. The reaction product was distributed between ether and dilute hydrochloric acid. The ether phase was washed and yielded 28.7 mg. of dry residue which was acetylated by treatment with 2 cc. of pyridine and 1 cc. of acetic anhydride at room temperature for 17 hours. The crude acetates (34.1 mg.) were isolated in the usual manner (2) and proved to be free of ketonic starting material (IV), as they gave no color with alkaline *m*-dinitrobenzene (18). Two recrystallizations from methanol furnished 13.9 mg. (39 per cent) of Δ^5 -pregnenetriol-3 β ,17 α ,20 α diacetate (I) which crystallized in elongated plates melting at 202–206°. Continued recrystallization raised the melting point to 204–206°. There was no depression on admixture of the isolated diacetate (m.p. 203–206°).

Analysis— $C_{28}H_{48}O_6$. Calculated, C 71.74, H 9.15; found, C 71.70, H 9.21

Rotation— $[\alpha]_D^{25} = -81^\circ$ ($c = 0.42$)

The infra-red spectrum is given in Curve A of Fig. 1.

The first mother liquor obtained in the preparation of diacetate I was chromatographed on alumina. The earlier eluates obtained with a 1:1 mixture of petroleum ether and benzene and with benzene (and the corresponding fractions obtained by rechromatographing later eluates) were crystallized from dilute ethanol and from petroleum ether-acetone and gave 7.0 mg. (20 per cent) of needles which showed incomplete fusion at 153–154° and melted after partial resolidification at 158°. The Δ^5 -preg-

nenetriol- $3\beta,17\alpha,20\beta$ 3,20-diacetate (V) was dried for analysis, rotation, and spectrographic measurements at 80° *in vacuo*.

Analysis— $C_{21}H_{30}O_5$. Calculated, C 71.74, H 9.15; found, C 71.82, H 9.19

Rotation— $[\alpha]_D^{25} = -27^\circ$ ($c = 0.54$)

The melting points recorded in the literature are 152 – 153° (uncorrected) (14), 159 – 160° (corrected) "nach leichtem Sintern bei 155° " (21) and 151 – 152° (17). The published rotations $[\alpha]_D = -36^\circ$ (alcohol) (14) and -34.7° (acetone) (21), while in good agreement with each other, show rather large differences from the rotations (8, 31, 9) of the reduction product, allopregnenetriol- $3\beta,17\alpha,20\beta$ 3,20-diacetate. The value found by us for the Δ^5 compound is more in harmony with the standard value for this transformation ($[\Delta[M]_D^{25}]^{5-\text{allo}} - \Delta^5$ for 3β -acetates in alcohol or acetone) and therefore may not be in error. The main maxima in the accessible finger-print region of infra-red radiation are at 13.87, 12.495, 12.32, 12.03, 11.35, 11.075, 10.66, 10.32, 10.21, 10.08, 9.67, 9.31, 8.79, 8.59, 8.31, 8.035, 7.78, 7.59, and 7.28 μ .

Δ^5 -Pregnenetriol- $3\beta,17\alpha,20\alpha$ (II)—16.2 mg. of synthetic diacetate (I), 32 mg. of sodium hydroxide, and 5 cc. of 80 per cent ethanol were heated under a reflux for 1 hour. The resulting triol (12.3 mg.) was isolated by ether extraction and crystallized from 95 per cent ethanol in elongated plates which melted at 221 – 224° with some browning. Admixture of Compound C caused no depression. The dried analytical specimen was analyzed without taking precautions against the uptake of water.

Analysis— $C_{21}H_{34}O_4 \cdot \frac{1}{2}H_2O$. Calculated, C 73.43, H 10.27; found, C 73.41, H 10.35

Δ^5 -Pregnenetriol- $3\beta,17\alpha,20\beta$ (VI)—6.8 mg. of diacetate V were hydrolyzed as described above. The free triol crystallized from acetone in heavy blocks that melted at 224 – 228° with decomposition. Butenandt *et al.* (14) reported a melting point of 227° (uncorrected) for their preparation.

Degradation of Δ^5 -Pregnenetriol- $3\beta,17\alpha,20\alpha$ (Synthetic) (II) and of Δ^5 -Pregnenetriol- $3\beta,17\alpha,20\beta$ (VI)—6.5 mg. of triol II and 2.0 mg. of triol VI were oxidized separately with periodic acid as described above. The product of each reaction when dissolved in carbon disulfide gave an infra-red spectrum in good accord with that of authentic dehydroisoandrosterone. The reaction product derived from triol II was benzoylated and gave a derivative melting at 254 – 258° with decomposition and at 254 – 258° in admixture with dehydroisoandrosterone benzoate. It was dried for analysis at 100° *in vacuo*.

Analysis— $C_{21}H_{28}O_3$. Calculated, C 79.55, H 8.22; found, C 79.54, H 8.29

SUMMARY

Compound C isolated from the non-ketonic digitonin-precipitable fraction obtained from the urine of a boy with an adrenocortical tumor has the structure of Δ^5 -pregnenetriol- 3β , 17α , 20α . It has been degraded with periodic acid to dehydroisoandrosterone and synthesized from Δ^5 -pregnenediol- 3β , 17α -one-20 acetate with lithium aluminum hydride. The known Δ^5 -pregnenetriol- 3β , 17α , 20β was also obtained, though in lesser amounts, from this reduction. The isolated triol is believed to be a metabolite of Δ^5 -pregnenediol- 3β , 17α -one-20. A brief account is given of the compounds isolated from the non-ketonic fraction which did not precipitate with digitonin.

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AMINO ACID DERIVATIVES IN BACTERIAL METABOLISM*

I. DERIVATIVES OF LEUCINE, PHENYLALANINE, TRYPTOPHAN, AND VALINE†

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The expanding use of microorganisms in the study of amino acid metabolism has brought with it many new and some as yet unsolved problems. One of the questions that is continually before the microbiological analyst is whether the growth response of an organism to a given amino acid is specific or whether some derivatives or closely related compound might serve just as well. Scattered observations have shown that in some cases peptides, keto acids, hydroxy acids, or other derivatives may be used in place of the corresponding amino acids by some bacteria (2-13).

This report is the first of a projected series dealing with the replacement of amino acids by certain of their derivatives and analogues in the growth of lactic acid bacteria.

EXPERIMENTAL

The synthetic medium, Table I, was a modification of that employed by McMahan and Snell (12). Microorganisms used were *Lactobacillus arabinosus* 17-5 (8014), *Lactobacillus casei* (7469), and *Leuconostoc mesenteroides* P-60 (8042). All cultures were obtained from the American Type Culture Collection of Georgetown University, Washington, D. C., and were maintained on microassay culture agar (Difco) by semimonthly transfers. Inocula for use in the tests were 18 hour cultures grown in microinoculum broth (Difco) which were washed twice by centrifugation in sterile isotonic phosphate buffer (pH 6.8). Finally the crop of organisms was suspended in enough of the sterile buffer to give a turbidity reading of 65 in an 18 mm. culture tube in a Coleman junior spectropho-

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† The author wishes to express his appreciation to Miss S. L. Cooley for a sample of acetyldehydrotryptophan and to Dr. John L. Wood for the derivatives of valine used in this study.

tometer at wave-length 660 m μ ; 0.05 ml. of this suspension was used per assay tube.

Conventional assay techniques were followed throughout, 10 ml. being the final culture volume, the substances under test being added to the medium before autoclaving. The incubation time was 72 hours at 37°. The acid produced was titrated with 0.10 N NaOH.

TABLE I
Composition of Basal Medium

Component	Amount*	Component	
	gm.		mg.
Glucose	40	Adenine sulfate	20
NaC ₂ H ₃ O ₂ (anhydrous)	12	Guanine·HCl	20
KH ₂ PO ₄	4	Uracil	20
K ₂ HPO ₄	4	Xanthine	20
(NH ₄) ₂ SO ₄	6	Choline	5
	mg.	Inositol	5
MgSO ₄ ·7H ₂ O	800		γ
MnSO ₄ ·H ₂ O	120	Biotin	10
NaCl	40	Folic acid	40
FeSO ₄ ·7H ₂ O	40	Riboflavin	400
CaCl ₂	55	<i>p</i> -Aminobenzoic acid	600
L-Asparagine	400	Ca pantothenate	800
L-Glutamic acid	4000	Nicotinic acid	1200
Other amino acids†		Pyridoxine·HCl	2400
L forms	200	Thiamine chloride	400
	400		

* Quantities used to prepare 1 liter of double strength medium. The pH was adjusted to 6.8.

† Alanine, arginine hydrochloride, aspartic acid, cystine, glycine, histidine hydrochloride, hydroxyproline, isoleucine, leucine, lysine hydrochloride, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

Amino acid derivatives and analogues were prepared according to the methods referred to in Table II.

RESULTS AND DISCUSSION

From the data recorded in Table II, it appears that several derivatives are quite capable of supporting growth of microorganisms in the absence of the free amino acid. The data reported here on leucine and valine are in accord with and extend the observations made by Hegsted (9) concerning the utilization of acetyl and keto derivatives of these amino acids by

L. arabinosus. The fact that the keto analogues support growth only to the extent of 50 per cent of the L form is still without further explanation. Our 72 hour incubation time apparently was not long enough to show the activity of D-leucine.

TABLE II
Microbiological Response to Amino Acid Derivatives

Derivative and bibliographic reference	Per cent activity*		
	<i>L. arabinosus</i>	<i>L. casei</i>	<i>L. mesenteroides</i>
L-Leucine	100	100	100
DL-Leucine.....	50	50	50
Acetyl-DL-leucine (14)	50	0	0
Chloroacetyl-DL-leucine (15)	50	0	0
Acetyldehydroleucine (16)	0	0	0
α -Ketoisocaproic acid (17)	50	50	0
L-Phenylalanine	100	100	100
DL-Phenylalanine	50	50	50
Acetyl-DL-phenylalanine (18)	0	0	0
Chloroacetyl-DL-phenylalanine (19).....	0	0	0
Acetyldehydrophenylalanine (20)	0	0	0
Phenylpyruvic acid (21), autoclaved	0-5†	0-5†	0
“ “ unautoclaved	100	100	25-35
L-Tryptophan	100	100	100
DL-Tryptophan	50	50	50
Acetyl-DL-tryptophan (22)	0	50	0
Chloroacetyl-DL-tryptophan (23).....	0	50	0
Acetyldehydrotryptophan	0	0	0
Indolepyruvic acid (24), autoclaved	0-5†	0-5†	0
“ “ unautoclaved	0-5†	0-5†	0
L-Valine	100	100	100
DL-Valine	50	50	50
Acetyl-DL-valine	0	0	0
Chloroacetyl-DL-valine	0	0	0
Acetyldehydrovaline	0	0	0
Dimethylpyruvic acid	50	50	0

* On a molar basis with L forms = 100.

† Variable growth reported in the literature (13); confirmed under our assay conditions.

‡ Slight growth at relatively high concentration of amino acid derivative.

With regard to the activity of the acetyl and chloroacetyl derivatives, enzymatic hydrolysis of these compounds, yielding the free amino acids, is highly possible and has been shown to occur in some systems (25). Since the chloroacetyl derivatives are utilized in two instances similarly to the acetyl, both are probably hydrolyzed prior to utilization inasmuch as

the chloroacetyl group is not considered a metabolic intermediate. Autoclaving did not appear to hydrolyze these compounds, as indicated by the failure of the medium to furnish the leucine requirement of *L. casei* and *L. mesenteroides* or the tryptophan requirement of *L. arabinosus* and *L. mesenteroides* in parallel experiments.

None of the various derivatives of phenylalanine tested was found to satisfy the phenylalanine requirements of the three organisms when employed under our test conditions. However, when phenylpyruvic acid was sterilized separately by Seitz filtration and added aseptically to the previously autoclaved test system, the derivative supported growth of the test organisms. Prescott *et al.* (13) have reported the variable response obtained in the presence of phenylpyruvic acid; this apparently is due to the variable destruction obtained when the compound is autoclaved in the test medium. Why *L. mesenteroides* used the unautoclaved phenylpyruvic acid to the extent of only 25 to 35 per cent is not apparent and is receiving further study. Possibly a longer incubation time would yield 100 per cent activity.

Tests with autoclaved and unautoclaved indolepyruvic acid did not reveal any growth-supporting properties of that compound. *L. casei* and *L. arabinosus* exhibited a small questionable response to relatively large amounts ($>100 \gamma$ per 10 ml. of medium) of indolepyruvic acid, whereas *L. mesenteroides* failed completely to grow under identical conditions. Slow conversion by the organisms may occur, but instability of the keto acid, even under great precaution, may be such that the compound was destroyed before the organisms could use it. Dimethylpyruvic and α -ketoisocaproic acids seem to be more stable to autoclaving than phenylpyruvic acid, since the activity found here corresponds to that found by Hegsted (9), who added the sterile keto acids after the medium was autoclaved.

It has been shown that certain acetyldehydroamino acids are not used by the rat for growth (24, 25-28). From our experimental data it appears that the acetyldehydroamino acids tested are also not effective in supporting growth of the microorganisms employed here.

It seems evident that bacterial organisms exhibit wide variations with respect to the utilization of amino acid derivatives. Each organism possesses its individual metabolic patterns, the character of which must be established by experimentation. Acetyl derivatives and keto analogues of amino acids already known to be utilizable by higher animals and to be present in various biological systems are now shown to support growth of certain microorganisms but not others. It is apparent that *L. mesenteroides* is more specific in its amino acid requirements than *L. arabinosus* or *L. casei*. Accordingly, it should afford assay results with greater valid-

ity. However, the availability of organisms which are known to respond in characteristically different manner to various derivatives introduces the possibility of identifying a biologically active derivative, even in the presence of the free amino acid. Unpublished data from this laboratory, involving additional organisms, indicate that the acetyl and keto derivatives of some amino acids may very well be differentiated from the amino acid and quantitatively estimated by the use of several organisms in parallel assay.

SUMMARY

As determined by the growth response (acid production) of the three microorganisms studied, the acetyl and chloroacetyl derivatives of DL-leucine can replace the DL-amino acid only for *L. arabinosus*; acetyl-DL- or chloroacetyl-DL-tryptophan is utilizable only by *L. casei*; the keto analogues of leucine and valine are only 50 per cent as active as the respective L-amino acids for both *L. arabinosus* and *L. casei*, and are not utilized at all by *L. mesenteroides*; the keto analogues of phenylalanine and tryptophan and the acetyldehydro derivatives of leucine, phenylalanine, tryptophan, and valine are non-utilizable under conditions of our experiments. None of the test organisms utilized for growth any of the phenylalanine derivatives under our conventional assay procedure. However, when phenylpyruvic acid was sterilized by Seitz filtration and added aseptically to the previously autoclaved test system, it supported growth of all three organisms.

Inasmuch as only one of the amino acid substitutes which we have employed will support the growth of *L. mesenteroides*, this organism appears more specific than *L. casei* or *L. arabinosus* in assays for leucine, tryptophan, and valine.

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THE DETERMINATION OF SULFHYDRYL GROUPS IN SERUM*

I. METHODS AND RESULTS ON NORMAL SERA

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Compounds containing sulfhydryl groups have occupied a unique position in metabolic and chemical studies because of their intimate association with the problems of protein structure (1), enzyme function (2), and cellular proliferation (3). Kolthoff and Harris (4) described an amperometric method for the determination of mercaptans which Benesch and Benesch adapted for use with amino acids and proteins (5). We have employed this method to investigate the sulfhydryl content of normal human serum, serum albumin, and serum globulin.

The details of the methods and results obtained on sera secured from normal individuals are presented at this time.

EXPERIMENTAL

Experience with the method of Benesch and Benesch (5) showed that revisions of the procedure were necessary to insure reproducibility of results. Other changes were made to permit the simultaneous determination of sulfhydryl and protein content (by the biuret and Kjeldahl methods) in the fractions derived from serum by the Pillemer and Hutchinson method (6). Because the platinum electrodes may respond erratically after varying periods of use, the electrode is standardized daily by means of a stock solution of a known mercaptan. Although cysteine hydrochloride was originally employed, we now prefer *n*-dodecyl mercaptan because of its greater stability.

Reagents—

n-Dodecyl mercaptan.¹ A stock standard is made by dissolving approximately 75 mg. in 10 ml. of absolute ethanol. 1 ml. of the stock solution

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¹ We wish to thank Dr. P. Tawney of the United States Rubber Company for the gift of *n*-dodecyl and *n*-tetradecyl mercaptans. *n*-Dodecyl mercaptan may be

is diluted to 25 ml. with absolute ethanol for use as the working standard. This solution contains approximately 0.00150 mm of SH per ml. and will require less than 2 ml. of 0.001 N silver nitrate for standardization.

0.001 N silver nitrate. A liter of 0.1 N silver nitrate solution is prepared from Mallinckrodt silver nitrate, analytic reagent. The silver nitrate is standardized against recrystallized sodium chloride (7). A liter of 0.001 N is made by dilution and stored in the dark reservoir of an automatic burette.

Supporting electrolyte. 30 gm. of ammonium nitrate are added to a volumetric flask containing 125 ml. of concentrated ammonium hydroxide and 100 ml. of water. The solution is brought to room temperature and made up to 250 ml.

Dilute methanol. 416 ml. of absolute methanol are diluted to 2 liters with distilled water and stored in a dispensing burette reservoir.

Biuret reagents. (a) A 1 per cent solution of copper sulfate pentahydrate; (b) a 5 per cent solution of sodium potassium tartrate in 5 per cent sodium hydroxide.

Apparatus—The titration assembly consists of the following elements: (a) a General Electric portable type galvanometer, catalogue No. 32-C-226-G-7, whose sensitivity is 0.012 μ a. per scale division; (b) a mercury reference electrode and rotating platinum electrode (4); (c) a 2 foot length of Tygon tubing, 7 mm. outside diameter, filled with saturated potassium chloride solution. An 8 cm. length of 7 mm. glass tubing serves as the dipping end of the salt bridge. A tightly rolled coil of Whatman No. 1 filter paper is inserted into the open end of the bridge. This plug is replaced daily; (d) a reduction gear box, electrode holder, and drive shaft taken from a stirring apparatus (Arthur H. Thomas (1931), catalogue No. 9238). We have fitted the drive shaft with a rubber friction ring and used a constant speed alternating current motor with a friction plate to drive the stirrer. This is a Redmond, type T model No. 4028, 60 cycle, 110 volt motor. The motor is mounted on a base plate which can be made to move laterally by rotating a knurled knob. This alters the position of the rubber drive ring on the friction drive plate mounted on the motor shaft, permitting a wide choice of speeds at constant torque. The rate of electrode revolution is not critical, provided the rate does not vary during a titration. We find that approximately 130 r.p.m. is a suitable rate; (e) an automatic 2 ml. burette (Scientific Glass, catalogue GG6-42, No. M570), with the reservoir painted black.

secured from the Paragon Division, Matheson Company, East Rutherford, New Jersey. It should be distilled *in vacuo* before use.

Methods

Electrode Standardization—1 ml. of dilute working standard is pipetted into a 100 ml. beaker containing 29 ml. of absolute ethanol and 1 ml. of supporting electrolyte. The electrode is started and the bridge opened. After a few minutes, the galvanometer comes to rest at its zero point, and the addition of silver nitrate is begun. After each addition of silver nitrate, one waits a few seconds for the galvanometer to register a steady state before taking the reading. This is important, since the first sign of

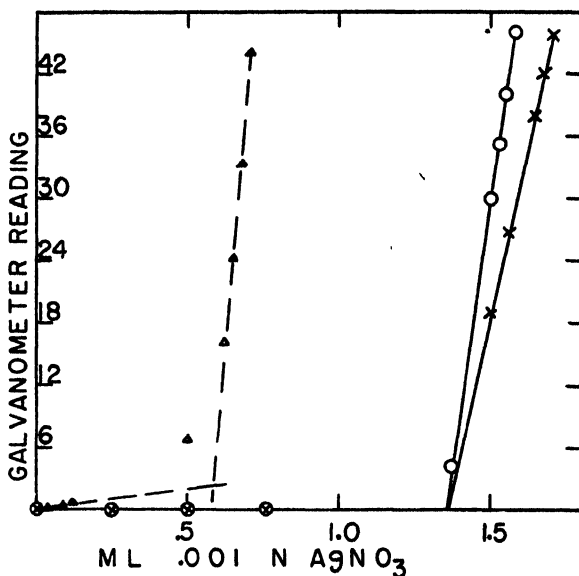


FIG. 1. Standardization curves of *n*-dodecyl mercaptan in 90 per cent ethanol (Curve X) and in 90 per cent methanol (Curve O). Titration of 1 ml. of Serum A (Δ). The serum contained 58.9 μ M of SH per 100 ml.

poor electrode response is an aimless drift of the galvanometer. Fig. 1 shows typical standardization curves for *n*-dodecyl mercaptan in 90 per cent ethanol and methanol.

Serum Titrations—Serum and serum fractions are titrated in dilute methanol in the same manner as the standard. 1 ml. of serum or the appropriate amount of serum fraction (see below) is added to a mixture of dilute methanol and supporting electrolyte until the final volume is 31 ml. in all cases. The titration is then carried out as above (Fig. 2). After each titration, the electrode and salt bridge are rinsed with 0.14 M saline followed by distilled water, and blotted dry with filter paper.

Preparation of Serum—25 to 30 ml. of blood from a fasting subject are drawn into dry tubes and allowed to clot at room temperature for 45 minutes. While the blood is clotting, the daily check of the electrode is carried out. The clots are rimmed with a glass rod and the tubes are centrifuged. The serum is poured into clean centrifuge tubes and again centrifuged for 5 minutes to remove any remaining cells. This procedure yields from 10 to 15 ml. of clear, non-hemolyzed serum. 6 ml. of serum are required for the serum fractionation, sulfhydryl, Kjeldahl, and biuret

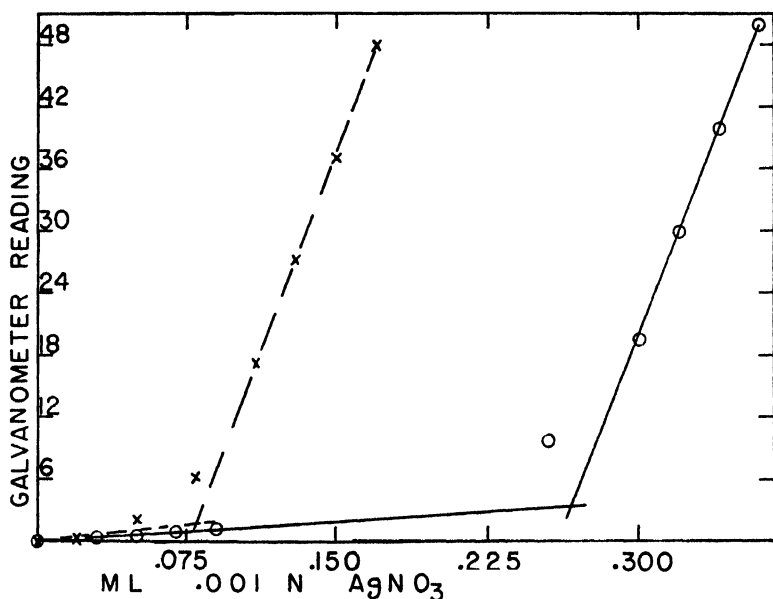


FIG. 2. Titration of Serum A fractions. Curve O, serum albumin, 4 ml. of aliquot. The albumin fraction contained $46.9 \mu\text{M}$ of SH per 100 ml. Curve X, serum globulin, 1 ml. of aliquot. The globulin fraction contained $11.9 \mu\text{M}$ of SH per 100 ml.

procedures. The remainder may be frozen and stored in a dry ice cabinet for electrophoretic, stability, and other studies.

The 6 ml. of serum are distributed as follows: Slightly more than 2 ml. are placed in a tube in a cold room at -1° for fractionation. While this sample is cooling, 1 ml. of serum is diluted to 25 ml. in a volumetric flask for use in the Kjeldahl and biuret determinations. Two 1 ml. samples are titrated for the determination of sulfhydryl in the whole serum.

Fractionation Procedure—2 ml. of serum which have been at -1° for at least 45 minutes are pipetted into a 15 ml. conical, graduated centrifuge

tube. The fractionation is conducted according to the method of Pillemer and Hutchinson (6) up to and including centrifugation at 3000 r.p.m. Following centrifugation, the albumin supernatant is carefully decanted into a graduated centrifuge tube and allowed to drain completely. The volume of this supernatant solution varies from 8.5 to 9.2 ml. Specimens from normal individuals have a range of 8.9 to 9.2 ml. Water is added to bring the volume to 14 ml. and the solution is well mixed, stoppered, and kept at -1° . The solution is brought out temporarily to room temperature for the removal of aliquots for the Kjeldahl, sulfhydryl, and biuret determinations.

The globulin precipitate, which has been drained as dry as possible, is dissolved by the addition of sufficient 0.14 M sodium chloride so that the final volume is 3 ml. In the event of an unusually large globulin fraction, it is brought to 4 ml. The globulin does not go completely into solution, maintaining a cloudy appearance. It should be well stirred before withdrawing aliquots for analysis.

Following the completion of the fractionation, aliquots are removed for Kjeldahl determination. Two 0.5 ml. samples of albumin and two 0.2 ml. samples of globulin are taken. 1 ml. and 0.2 ml. Mohr pipettes graduated in 0.01 ml. divisions are used. At the same time, two 1 ml. samples of diluted whole serum (see "Preparation of serum") are taken.

While the Kjeldahl samples are digesting, the serum fractions are titrated for sulfhydryl. The albumin is determined by adding 4 ml. of the 14 ml. supernatant to a beaker containing 26 ml. of dilute methanol and 1 ml. of supporting electrolyte. For the globulin determination, 1 ml. aliquots are used if the final volume was 3 ml. or 1.5 ml. if the final volume was 4 ml. These are added to 29 ml. of dilute methanol plus 1 ml. of electrolyte with the electrode stirring. At this point, the globulin generally forms a clear solution. Both fractions are titrated in duplicate. The samples are kept at -1° between runs.

Following the sulfhydryl titrations, the biuret determinations are made. Duplicate samples are prepared as follows: 1 ml. of albumin and 7.0 ml. of water; 0.2 ml. of globulin and 7.8 ml. of water; 2.0 ml. of dilute serum and 6.0 ml. of water.

To these tubes are added, with rotary agitation, 1 m $\bar{f}$. of the alkali and 1 ml. of copper²⁺sulfate. After standing at least 5 minutes, they are read at 560 λ in a Coleman model No. 14 spectrophotometer, with round cuvettes having a light path of 15.7 mm.

Calculations—1 ml. of 0.001 N silver nitrate is equivalent to 1 μ M (micromole) of SH. The results to be presented are given in terms of micromoles of SH per 100 ml. of serum or micromoles per gm. of nitrogen.

This method of presentation is independent of the sulfhydryl compound used as a standard and does not arbitrarily assign the sulfhydryl group to the cysteine molecule.

Total serum sulfhydryl is given by (a).

$$(a) \quad \frac{\text{Ml. } 0.001 \text{ N AgNO}_3}{\text{Ml. serum}} \times \frac{100}{1} = \frac{\mu\text{M SH}}{100 \text{ ml. serum}}$$

Albumin sulfhydryl is given by (b).

$$(b) \quad \frac{\text{Ml. } 0.001 \text{ N AgNO}_3}{4} \times \frac{14}{2} \times \frac{100}{1} = \frac{\mu\text{M albumin SH}}{100 \text{ ml. serum}}$$

Globulin sulfhydryl is given by (c) or (c').

$$(c) \quad \frac{\text{Ml. } 0.001 \text{ N AgNO}_3}{1} \times \frac{3}{2} \times \frac{100}{1} = \frac{\mu\text{M globulin SH}}{100 \text{ ml. serum}}$$

$$(c') \quad \frac{\text{Ml. } 0.001 \text{ N AgNO}_3}{1.5} \times \frac{4}{2} \times \frac{100}{1} = \frac{\mu\text{M globulin SH}}{100 \text{ ml. serum}}$$

The biuret values are

$$(d) \quad \frac{D}{2} \times \frac{25}{1} \times \frac{100}{1} = \frac{D}{100 \text{ ml. serum}}$$

$$(e) \quad \frac{D \text{ albumin}}{1} \times \frac{14}{2} \times \frac{100}{1} = \frac{D \text{ albumin}}{100 \text{ ml. serum}}$$

$$(f) \quad \frac{D \text{ globulin}}{0.2} \times \frac{3}{2} \times \frac{100}{1} = \frac{D \text{ globulin}}{100 \text{ ml. serum}}$$

$$(f') \quad \frac{D \text{ globulin}}{0.2} \times \frac{4}{2} \times \frac{100}{1} = \frac{D \text{ globulin}}{100 \text{ ml. serum}}$$

D = optical density.

The correlation values for peptide to nitrogen, $D/(\text{mg. of N})$, and sulfhydryl to peptide, SH/D , were derived by using the above values and the Kjeldahl nitrogen content for the appropriate fraction or whole serum. No correction was made for non-protein nitrogen in normal sera.

Electrophoretic analyses were performed in a modified Longsworth apparatus.² All determinations were carried out in veronal buffer, pH 8.6, 0.1 ionic strength at 1°.

² The electrophoresis apparatus was modified by Dr. H. Sidney Newcomer of New York so as to fold the optical path on itself. This has been accomplished by the

Results

Table I shows the distribution of sulfhydryl and nitrogen in the serum proteins of normal males and females. It is apparent that there is no

TABLE I
Nitrogen and Sulfhydryl Content of Human Sera and Serum Fractions

Subject (a)	Age (b)	Total N as gm. per 100 ml. serum (c)			μ M SH per 100 ml. serum (d)			μ M SH per gm. N (e)		
		Serum	Albu- min	Glob- ulin	Serum	Albu- min	Glob- ulin	Serum	Albu- min	Glob- ulin
Normal males										
E. S.	37	1.23	0.69	0.45	56.0	47.8	12.3	45.6	69.4	27.3
N. W.	34	1.22	0.64	0.51	52.4	39.6	11.4	43.0	62.0	22.4
J. G.	24	1.15	0.71	0.43	54.0	45.5	11.0	47.0	64.1	25.6
J. C.	34	1.15	0.67	0.43	53.1	44.3	10.5	46.2	66.1	24.4
M. L.	29	1.25	0.70	0.50	52.9	42.4	12.0	42.3	60.6	24.0
L. S.	25	1.26	0.78	0.44	53.2	46.4	10.4	42.2	59.5	23.7
D. B.	21	1.26	0.73	0.50	59.0	47.1	12.3	46.8	64.5	24.6
J. B.	27	1.09	0.70	0.37	52.0	45.0	9.2	47.7	64.3	24.9
R. P.	28	1.18	0.75	0.38	52.8	46.4	9.3	44.7	61.8	24.5
Mean		1.20	0.71	0.45	53.9	44.9	10.9	45.1	63.6	24.6
σ		0.06	0.04	0.05	2.2	2.6	1.2	2.1	3.0	1.4
Normal females										
D. F.	23	1.21	0.71	0.46	55.6	47.2	9.9	45.9	66.5	21.5
B. A.	23	1.17	0.66	0.46	53.5	41.2	10.4	45.7	62.4	22.6
K. J.	24	1.15	0.70	0.51	52.8	43.4	12.5	45.9	62.0	24.5
M. H.	28	1.24	0.68	0.51	48.8	40.1	10.7	39.3	59.0	21.0
J. F.	24	1.23	0.66	0.54	51.0	41.7	12.0	41.5	63.2	22.2
J. P.	31	1.18	0.73	0.40	55.8	47.4	10.4	47.3	64.9	26.0
A. M.	24	1.20	0.67	0.49	53.1	45.0	11.6	44.3	67.2	23.7
H. P.	27	1.18	0.71	0.42	50.8	45.0	9.9	43.0	63.4	23.6
B. B.	23	1.19	0.69	0.45	52.5	45.3	10.5	44.1	65.7	23.3
C. L.	24	1.13	0.65	0.44	52.0	42.7	10.5	46.0	65.7	23.9
Mean		1.18	0.69	0.47	52.6	43.9	10.8	44.3	64.0	23.2
σ		0.04	0.03	0.04	2.1	2.5	0.9	2.4	2.5	1.5

σ = standard deviation $\times \sqrt{n/(n-1)}$.

significant difference between the sexes. These subjects were drawn from a university hospital staff population and have a restricted age distribution. The use of penta mirrors and prisms, and will be described in a forthcoming publication.

tion.³ The ratio of albumin nitrogen to globulin nitrogen is the same as that reported for normal sera by Pillemer and Hutchinson (6).

From the data presented in Column *d*, Table I, it may be seen that albumin SH represents 80 per cent of the total serum SH. It is known that alterations in the albumin and globulin contents of serum occur in many pathological states (8). Thus it might be anticipated that the SH values of pathological sera would be found altered. This is indeed the case, as will be shown in a subsequent paper.⁴

Evidence concerning a difference in the composition of the two fractions is tabulated under Column *e*. There is approximately $2\frac{1}{2}$ times as much SH per gm. of albumin nitrogen as there is per gm. of globulin nitrogen. While sufficient data are not available to evaluate this observation completely, some information may be deduced from reported analyses of purified proteins (9). If we assume a molecular weight of 69,000 and 16.0 per cent of nitrogen, there would be 11,040 gm. of nitrogen per mole of human serum albumin. Our value of 64 μM of SH per gm. of serum albumin nitrogen results in a value of 0.71 mole of SH per mole of albumin. Hughes has isolated a crystalline mercury derivative of human albumin as the compound albumin-Hg-albumin (10). His data indicate that 0.67 mole of SH per mole of serum albumin is present. It would appear that the SH groups titrated amperometrically are the same as those yielding the crystalline mercury salt. Brand has reported 4 moles of cysteine per mole of human serum albumin (9). In view of the findings of Olcott and Fraenkel-Conrat that tryptophan reacts with cystine to give cysteine, it is not certain what value can be attached to the reported cysteine contents, determined on acid hydrolysates (11).

The globulin fraction of normal human serum includes α -, β -, and γ -globulins in approximately equal concentration. The average molecular weight of the human serum globulins has been taken as 170,000 (12). If one assumes an average nitrogen content of 15 per cent, then the 24 μM of SH per gm. of globulin nitrogen determined would be equivalent to 0.6 mole of SH per average globulin molecule. This value approximates that found for albumin, but its distribution among the different globulins is not known.

The biuret reaction may be employed as a measure of the number of peptide linkages in the serum proteins. Table II presents some interesting observations with regard to the relationship of peptide bonds to sulfhydryl groups in the serum proteins. It may be seen (*a*) that the ratio

³ We have determined the SH in fetal serum. The values for micromoles of SH per 100 ml. of serum are lower than in adults. However, μM SH/D is the same as in adult serum.

⁴ Schoenbach, E. B., Weissman, N., and Armistead, E. B., to be published.

of peptide bonds per mg. of nitrogen is nearly alike for serum albumin and globulin. Since the albumin fraction contains the non-protein nitrogen, one would expect the slightly lower biuret extinction found per unit of nitrogen. In contrast to this similarity, (b) the albumin fraction is

TABLE II
Correlation of Peptide and Sulfhydryl Content of Serum Proteins

Subject	$\frac{D}{\text{mg. N}}$			$\frac{\mu\text{M SH}}{D}$			Albumin Globulin		
	(a)			(b)			(c)		
	Serum	Albu- min	Glob- ulin	Serum	Albu- min	Glob- ulin	Biuret	Kjel- dahl	Electro- phoresis
Males									
E. S.	0.244	0.244	0.267	0.187	0.283	0.103	1.42	1.53	1.38
N. W.	0.246	0.261	0.254	0.175	0.237	0.088	1.29	1.26	1.17
J. G.	0.243	0.234	0.256	0.193	0.276	0.101	1.51	1.65	
J. C.	0.256	0.251	0.290	0.181	0.262	0.085	1.36	1.56	
M. L.	0.258	0.252	0.283	0.164	0.241	0.086	1.26	1.40	
L. S.	0.228	0.244	0.254	0.185	0.244	0.094	1.71	1.77	
D. B.	0.236	0.235	0.262	0.198	0.277	0.093	1.29	1.46	
J. B.	0.241	0.236	0.265	0.198	0.271	0.093	1.68	1.89	
R. P.	0.233	0.237	0.257	0.192	0.261	0.096	1.84	1.97	
Mean	0.243	0.244	0.265	0.186	0.261	0.093	1.48	1.61	
σ	0.0099	0.0093	0.0130	0.0112	0.0171	0.0062	0.21	0.23	
Females									
B. A.	0.244	0.247	0.278	0.187	0.251	0.081	1.27	1.44	1.17
K. J.	0.250	0.233	0.267	0.183	0.265	0.092	1.21	1.37	1.17
M. H.	0.238	0.239	0.258	0.165	0.248	0.082	1.24	1.33	
J. F.	0.240	0.237	0.262	0.173	0.269	0.084	1.09	1.22	
J. P.	0.233	0.231	0.268	0.203	0.280	0.098	1.60	1.83	
A. M.	0.250	0.248	0.264	0.176	0.269	0.091	1.30	1.37	
H. P.	0.233	0.231	0.261	0.185	0.273	0.091	1.51	1.69	
B. B.	0.261	0.256	0.282	0.169	0.256	0.083	1.39	1.53	
C. L.	0.241	0.232	0.259	0.190	0.283	0.091	1.31	1.48	
Mean	0.243	0.239	0.267	0.181	0.266	0.088	1.32	1.47	
σ	0.009	0.009	0.008	0.012	0.012	0.018	0.16	0.19	

shown to contain 3 times as many sulfhydryl groups as the globulin fraction when these fractions are compared with reference to their peptide content.

A comparison of the albumin-globulin ratios (c) shows that the biuret values agree closely with the electrophoretic determinations. With one exception, the ratios of the biuret values are all lower than the Kjeldahl,

a reflection of the small non-protein nitrogen content of the albumin fraction, which increases the Kjeldahl ratio.

The effect of storage on the SH content was studied on nineteen serum samples. Serum stored at 0° showed a 6 per cent reduction after 24 hours, while at -78° no appreciable change was noted up to 4 months. Dialysis at 0° had little effect when compared to stored controls.

Effect of Certain Compounds of Biochemical Interest on Sulfhydryl Determination

When *n*-propylthiouracil was added to a serum whose SH content had been determined, it was recovered almost quantitatively. Ennor and Stocken have reported that sulfhydryl compounds interfere with the determination of creatine by the Barritt reaction (13). There was no change in the SH content of serum to which an equimolar amount of creatine was added. The addition of 0.1 to 1.0 mg. of sodium desoxyribonucleate to 1 ml. of serum also had no effect. Penicillin G gave a blank titration and, when added to serum, did not change the SH content. The observations of Benesch and Benesch (5) that *p*-chloromercuribenzoate completely abolished the SH of serum samples were confirmed.⁶

DL-Cysteine as well as *n*-dodecylmercaptan could not be titrated in dilute alcoholic solutions or when added to serum. L-Cystine, titrated in 90 per cent ethanol, showed no evidence of an SH group.

Sulfhydryl Content of Other Body Fluids

Plasma—Samples of serum and heparinized plasma were prepared from the same blood samples. The sulfhydryl values per ml. agreed within experimental error.

Saliva—1, 5, and 7 ml. samples of saliva were titrated, but no sulfhydryl groups were detected. The biuret determinations on these salivas indicated approximately 1.3 mg. of protein per ml.

Ascitic and Pleural Fluid—Ascitic and pleural fluid contain titratable SH. The SH/D values found in three samples were 0.145, 0.140, and 0.097. The serum value corresponding to the last value was 0.122. One ascitic fluid, which showed no SH, had so little protein that the sulfhydryl titration was considered unreliable.

Urine—A specimen of protein-free urine titrated as a blank.

DISCUSSION

The studies presented herein have been concerned with the protein sulfhydryl groups of serum. The concordance of values secured in sera be-

⁶ We wish to thank Dr. L. Hellerman for the gift of highly purified *p*-chloromercuribenzoate.

fore and after dialysis supports this assertion. These results are remarkable in view of the much higher content of sulfhydryl groups in the formed elements of the blood. The glutathione content of human erythrocytes⁶ is 35 mg. per 100 ml. of whole blood equivalent to 114 μM of SH (14). Thioneine has been reported present in human erythrocytes in amounts averaging 15 mg. per 100 ml. of whole blood or 65 μM of SH (15). Waelsch and Rittenberg have estimated that half of the glycine or of the glutamic acid of liver glutathione of rats and rabbits is replaced in less than 4 hours (17, 18). This is one of the most rapid turnover rates ever reported. These intracellular sulfhydryl compounds should be in equilibrium with serum non-protein sulfhydryl, yet our findings give no indication of such an equilibrium.⁷ It may be significant that Eagles and Vars were unable to detect thioneine added to blood or solutions of proteins. These authors observed that the thioneine seemed to be bound to protein (19). Also, it should be noted that both thioneine and glutathione have been isolated from cells only by very drastic chemical methods (16).

It is believed that the relationship between the ratio of biuret or peptide values to the nitrogen of the serum proteins $D/(\text{mg. of N})$ and the ratio of the sulfhydryl to peptide bond content of these proteins, micro-moles SH/ D , is of importance in evaluating the nature of the serum proteins. It would appear that there is no great difference between albumin and globulin in their number of peptide bonds per mg. of nitrogen. By contrast, the large difference in the number of sulfhydryl groups in these protein fractions on the basis of their peptide content is most striking. Luetscher found two components in human serum albumin by electrophoresis at pH 4. The faster of these was present to the extent of 67 per cent, and decreased markedly in the pathological sera examined (20). The mercaptalbumin isolated by Hughes contains 1 mole of SH per mole of protein (10). The 0.71 mole of SH per mole of albumin protein which we have found may represent a statistical average; *i.e.*, a mixture of albumins only one of which possesses a sulfhydryl group. It is possible that all newly formed albumin contains 1 mole of SH, which is lost with age.⁸ The hypothesis may be advanced that the lowered SH found in

⁶ The contribution of the leucocytes and platelets has not been measured (14-16). Benedict and Gottschall drew attention to the correlation between the hemoglobin and glutathione contents of the human bloods analyzed by them.

⁷ Studies on hemolyzed human erythrocytes have shown that there are about 1175 μM of intracellular SH per 100 ml. of whole blood. Of this, less than 6 per cent or 65 μM of SH are dialyzable. This latter amount is far below the total for glutathione and thioneine. Our results indicate that there are 4 to 5 moles of SH per mole of hemoglobin. Further work on this problem is in progress.

⁸ In a personal communication, Dr. Hughes has informed the authors that this theory is under test at Harvard.

pathological sera (21) represents an accelerated aging process, a retarded synthesis of new protein, or a rearrangement of molecular architecture. The globulin fraction will require separation into its component units before its sulfhydryl status becomes clear.

The manner in which sulfhydryl groups exist in proteins is unknown. In order to account for the appearance of SH groups, previously non-detectable, upon denaturation of crystalline ovalbumin, Harris postulated that there may be several forms of sulfur linkage other than S—S in native proteins (22). These postulated forms were thiopeptides, thioesters, and thiolactones. Walker showed that S—S linkages were not present in native ovalbumin (23). Mastin and Schryver studied the formation of an acidic denatured egg albumin and noted an increase in the number of free acid groups without a corresponding increase in amino groups. At the same time, free SH groups appeared. They considered this evidence for the existence of a thioester linkage in proteins (24). On the basis of very careful amino acid analyses, Chibnall has also postulated the existence of such protein linkages (1).

Greenstein found that, although guanidinium salts and urea produced SH groups in proteins, they had no such effect on a series of simple dithio compounds. He concluded that the sulfur in proteins which appeared as SH did not come from S—S (25). In our study, cystine in alcoholic solution did not titrate amperometrically.

It seems reasonable to infer that the "masked" sulfhydryl groups in proteins are actually linked in a chemical union which can be described in classical organic terms. It remains to be determined which mode or modes of linkage are most probable.

We wish to thank Dr. W. Mansfield Clark and Dr. Leslie Hellerman for helpful criticism and Mr. Jerry Reik for his aid in the performance of some of the electrophoretic analyses.

SUMMARY

1. Methods and details are given for the amperometric titration of the sulfhydryl group in human serum, serum albumin, and the serum globulin fraction.

2. The sulfhydryl contents of normal serum, serum albumin, and the serum globulin are presented.

3. The relationship of the sulfhydryl group to the number of peptide bonds has been determined. It is shown that normal serum albumin and the normal serum globulin fraction each have a characteristic ratio of sulfhydryl to peptide bond content.

4. Normal human serum albumin has 0.71 mole of sulfhydryl per mole

of protein. The implications of this finding with respect to the composition of albumin are discussed.

5. It is shown that the dithio linkage of cystine cannot be titrated amperometrically. The nature of sulfur linkages in proteins is discussed in the light of this and other observations.

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ON THE UTILIZATION OF L- AND D-ALANINE FOR THE ACETYLATION REACTION IN VIVO*

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Bloch and Rittenberg (1, 2) have reported that deuterio-DL-alanine and acetylamino acids labeled with deuterium in the acetyl group give rise to a large isotope concentration in the acetyl groups of amines containing an α -hydrogen atom, in contrast to amines not having an α -hydrogen atom. With deuterioacetic acid itself, no such difference in the acetylation of the two types of foreign amines was found, while deuteriopyruvic acid gave qualitatively similar though lower values than DL-alanine. Bloch and Rittenberg suggested that pyruvic acid, arising by deamination of alanine, could condense directly with phenylaminobutyric acid by the mechanism proposed by Knoop (3) and others (4) and that acetylation of *p*-aminobenzoic acid, on the other hand, took place only after conversion of the labeled pyruvic acid to acetic acid.

In a previous communication (5), differential acetylation of the two types of amines with carbon-labeled pyruvic acid was observed in a laboratory strain of rats of unknown origin.¹ In the Sprague-Dawley strain, on the other hand, it was found that the acetyl groups of the two types of amines had identical isotope concentrations after administration of carbon-labeled pyruvic acid (5). This has now been found to be the case also with three additional strains of rats (Table I).

The ability of carbon-labeled DL-lactic acid to serve as a precursor for the acetyl groups of the two kinds of amines has also been tested and compared to that of pyruvic and acetic acids. The resulting isotope concentrations (Table II) are identical in both types of acetylaminines.

Since the data obtained with carbon-labeled pyruvic and lactic acids were in disagreement with the results of Bloch and Rittenberg with deuterio-DL-alanine, acetylation experiments with carbon- and deuterium-labeled L- and D-alanine were carried out.

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† Aided by a grant from the United States Public Health Service.

¹ In view of the results reported here the data obtained with this strain indicate that its behavior is exceptional. In particular the use of differential acetylation for the calculation of the size of the pyruvic acid pool does not seem to be justified any longer.

EXPERIMENTAL

The synthesis of 2-C¹⁴-pyruvic acid has been reported previously (6).

2-C¹⁴-DL-Lactic Acid—This compound was prepared by hydrogenation of 2-C¹⁴-pyruvic acid in aqueous solution with hydrogen and Adams' catalyst at atmospheric pressure and room temperature.

TABLE I

Acetylation of Foreign Amines by 2-C¹⁴-Pyruvic Acid in Various Rat Strains

Rat strain	Dose (D.) of 2-C ¹⁴ pyruvic acid adminis- tered	Acetyl groups of			
		Acetylphenylamino- butyric acid		Acetyl- <i>p</i> -amino- benzoic acid	
		R. i. c.*	R. i. c. D.	R. i. c.*	R. i. c. D.
	<i>mM per 100 gm. per day</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Sprague-Dawley ..			2.5†		2.6†
Wistar ..	0.98	3.2	3.2	3.0	3.0
Sherman ..	1.0			2.8	2.8
Yale ..	0.74			2.8	3.8

* The relative isotope concentration is calculated for 100 per cent isotope in the administered compound.

† Average of several experiments.

TABLE II

Acetylation of Foreign Amines by 2-C¹⁴-DL-Lactic, 2-C¹³-Pyruvic, and Deuterioacetic Acids in Same Animal

Compound	Dose	Acetyl groups of			
		Acetylphenylamino- butyric acid		Acetyl- <i>p</i> -amino- benzoic acid	
		R. i. c.*	R. i. c. D.	R. i. c.*	R. i. c. D.
	<i>mM per 100 gm. per day</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
2-C ¹⁴ -DL-Lactic acid. . . .	1.02	1.1	1.1	1.1	1.1
2-C ¹³ -Pyruvic "	1.07			2.5	2.3
Deuterioacetic "	1.02	3.3	3.2	3.8	3.7

* See foot-note to Table I.

2-C¹⁴-D- and L-Alanines—2-C¹⁴-Pyruvic acid was treated with an excess of phenylhydrazine hydrochloride. The hydrazone was obtained in 95 per cent yield and hydrogenated quantitatively to DL-alanine with Adams' catalyst and hydrogen at atmospheric pressure and room temperature in water suspension. Alkali was added, the aniline formed during hydrogenation removed by extraction with ether, and the aqueous layer treated

with benzoyl chloride. After dilution with normal and deuteriobenzoyl-DL-alanine (1),² the optical antipodes were separated via the brucine and strychnine salts by the method of Fischer (7). The alkaloid salts were recrystallized to constant isotope concentration. D-Alanine, $[\alpha]_D = -12.9^\circ$, 15,700 c.p.m. as BaCO_3 , 9.3 per cent D; L-alanine, $[\alpha]_D = +12.5^\circ$, 20,600 c.p.m. as BaCO_3 , 9.5 per cent D.

The feeding experiments and the isotope analyses were carried out as previously described (5).

TABLE III

Acetylation of Foreign Amines by L- and D-Alanine in Rats of Sprague-Dawley Strain

Compound	Dose	Acetyl groups of			
		Acetylphenylamino- butyric acid		Acetyl-p-amino- benzoic acid	
		R. i. c.*	R. i. c. D.	R. i. c.*	R. i. c. D.
	mm per 100 gm. per day	per cent	per cent	per cent	per cent
2-C ¹⁴ -L-Alanine	0.93	1.1	1.1	1.1	1.2
“	0.96	1.8	1.9	1.1	1.2
“	0.41	0.92	2.2	0.49	1.2
“	0.48	0.68	1.4	0.49	1.0
Deuterio-L-alanine	0.48	0.12	0.25		
2-C ¹⁴ -D-Alanine	0.52	5.4	10.5	0.35	0.7
Deuterio-D-alanine	0.52	4.3	8.3	0.32	0.6
“	0.3	3.0	10.0		

* See foot-note to Table I.

Results

Results similar to those observed with pyruvic acid were obtained with L-alanine (Table III). The lower absolute values may be ascribed to dilution of the ingested L-alanine by L-alanine present in the tissues. D-Alanine, on the other hand, yielded a substantially higher isotope concentration in the acetyl group when phenylaminobutyric acid was administered than when p-aminobenzoic acid was given. The absolute isotope concentration in acetyl-p-aminobenzoic acid is of the same order of magnitude after D-alanine as after L-alanine administration, indicating that acetylation of the aromatic amine may well proceed by way of pyruvic and acetic acids. The difference in the ability of the optical antipodes of alanine to acetylate foreign amines containing an α -hydrogen atom accounts for the results obtained by Bloch and Rittenberg, and the results obtained with labeled pyruvic acid and L-alanine make acetylation of foreign amines *in vivo* by the Knoop mechanism unlikely.

² The deuterio-DL-alanine was kindly supplied by K. Bloch.

An experiment was carried out to investigate the possibility that D-alanine condenses with a keto acid to form an acetylamino acid after decarboxylation. This acetylamino acid could then transfer its acetyl group to a foreign amine with an α -hydrogen atom by a mechanism involving acetyl transfer, which has been shown to yield high isotope concentrations in acetylphenylaminobutyric acid (2). Unlabeled D-alanine was fed, together with labeled pyruvic acid. If these two compounds would condense to form acetylalanine, the double bond of the intermediary Schiff base would shift rapidly between the nitrogen and the two α -carbon atoms. The isotope would therefore be distributed in both the alanine and acetyl moieties and transacetylation would be expected to yield a higher isotope concentration in acetylphenylaminobutyric acid. The ex-

TABLE IV

Acetylation of Foreign Amines by Labeled Pyruvic Acid in Presence of Unlabeled D-Alanine

Compound	Dose	Acetyl groups of			
		Acetylphenylamino- butyric acid		Acetyl-D-amino- benzoic acid	
		R. i. c.*	$\frac{R. i. c.}{D.}$	R. i. c.*	$\frac{R. i. c.}{D.}$
	<i>mm per 100 gm. per day</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
2-C ¹³ -Pyruvic acid.....	0.56	0.9	1.6	1.2	2.1
+ D-alanine	2.5				

* See foot-note to Table I.

periment, however, gave identical isotope concentrations in both acetyl-amines (Table IV).

DISCUSSION

The results obtained with labeled pyruvic and lactic acids can be accounted for by assuming that most strains of rats are able to convert these acids rapidly to acetic acid, since, on an equimolar basis, acetic acid is 2 to 5 times as efficient in the acetylation reaction as are the 3-carbon compounds.

It appears that the unnatural form of alanine acetylates amines with an α -hydrogen atom more efficiently than amines which do not have an α -hydrogen atom. So far, no reaction in mammalian tissue is known which specifically utilizes an unnatural amino acid. This result, however, can be explained by the following mechanism.

It is postulated that acetic acid or an active derivative of unknown

constitution is the only immediate acetylating agent and that the sites of the acetylation reaction are different for the two kinds of foreign amines. For example, there is reason to assume that *p*-aminobenzoic acid is acetylated in the liver only. It has actually been observed, in experiments with tissue slices *in vitro*, that liver is the only organ which acetylates sulfanilamide (8) which is also an aromatic amine. Any labeled compound other than acetic acid then will provide acetyl groups for the acetylation of this amine only to the extent to which it is converted to acetic acid in the liver. The labeled acetic acid formed is diluted by the large hepatic pool. This assumption is in agreement with all of the experimental data, since acetic acid itself is the most efficient precursor for the acetyl group of *p*-aminobenzoic acid.

On the other hand, it may be assumed that phenylaminobutyric acid can be acetylated not only in the liver but also in the kidney. Experiments *in vitro* (9) support this hypothesis. Therefore, any labeled compound which is not completely metabolized in the liver and which, after transport to the kidney, can be converted in this organ to acetic acid will supply acetyl groups with high isotope concentrations for this amine, provided the dilution of the labeled metabolites in the kidney is smaller than in the liver. The experimental results with D-alanine can be explained adequately by this hypothesis, since the kidney has been shown by experiments *in vitro* to contain high concentrations of D-amino acid oxidase, which converts D-alanine to pyruvic acid (10), and an enzyme system which is capable of converting pyruvic acid to acetic acid (11). This hypothesis can also account for the fact that the average of the values of relative isotope concentration to dose for acetylphenylaminobutyric acid after administration of labeled L-alanine is about 1.5 times greater than the average values obtained for acetyl-*p*-aminobenzoic acid (Table III), if it is assumed that some of the administered L-alanine reaches the kidney and is utilized there in the acetylation reaction after deamination and conversion to acetic acid. This is in contrast to the results obtained with labeled pyruvic acid where the values for the two foreign amines are always found to be identical, indicating that any pyruvic acid reaching the kidney was diluted in the liver.

The results reported here therefore appear to indicate that *N*-acetylamines are synthesized *in vivo* by only one mechanism and that the differences observed with labeled precursors are due to different sites of acetylation of the two types of amines. If this is the case, acetylation of phenylaminobutyric acid in the kidney can also account for the results obtained by Bloch and Rittenberg (2) with various acetyl amino acids as sources for acetyl groups of foreign amines. The part of the acetyl amino acids not hydrolyzed in the liver provides acetyl groups with high isotope

concentrations in the kidney. An enzyme which hydrolyzes acetyl-L-amino acids (12) has been demonstrated to be present in high concentration in this organ. This alternative interpretation makes unnecessary the assumption of a special mechanism which transfers *N*-acetyl groups directly from one α -amino acid to another.

SUMMARY

Labeled pyruvic acid affords identical isotope concentrations in the acetyl groups of excreted acetylamines both with and without an α -hydrogen atom in four strains of rats *in vivo*. Labeled DL-lactic acid gave the same results as did pyruvic acid.

Labeled L-alanine is utilized as a precursor for acetyl groups in a similar manner as pyruvic acid, while labeled D-alanine gives rise to exceptionally high isotope concentrations in acetylphenylaminobutyric but not in acetyl-*p*-aminobenzoic acid. A possible mechanism to account for these results is discussed.

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TRANSAMINATION FROM GLUTAMINE TO α -KETO ACIDS*

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The striking activation of the deamidation of glutamine in rat liver extracts by pyruvic acid was first reported by Greenstein and Carter (2) and has been the subject of several subsequent investigations (3-7). This phenomenon was observed with pyruvic, phenylpyruvic, and α -ketoisocaproic acids, but not with pyruvoylglycine, phenylpyruvoylglycine, lactic acid, or γ -ketovaleric acid. The enzyme which catalyzes the α -keto acid activation of glutamine deamidation was separated from phosphate-activated glutaminase and partially purified by Errera (6). As an approach to an understanding of the mechanism of this interesting keto acid effect, it seemed of importance to investigate the specificity requirements of the system with respect to the keto acid. In the course of these studies, an apparently new type of transamination reaction has been observed, involving the transfer of the α -amino group of glutamine to a number of α -keto acids. The results of an investigation of this reaction, which is associated with the deamidation of glutamine, form the basis of this report.

EXPERIMENTAL

Materials—L-Glutamine was obtained from the Nutritional Biochemicals Corporation. When boiled with 2 N hydrochloric acid for 1 minute, 95 per cent of the theoretical amide nitrogen was recovered. Synthetic L-glutamine, prepared according to Bergmann *et al.* (8), and L-glutamine, labeled with N¹⁵ in the amide group (9), were donated by Dr. M. Berenbom. The normal α -keto acids were obtained and characterized as described previously (10). Sodium α -ketoisocaproate, L-isoglutamine, acetyl-DL-glutamic acid, D-alloisoleucine, and the L isomers of methionine, glutamic acid, leucine, alanine, α -aminobutyric acid, and tyrosine were donated by Dr. J. P. Greenstein. L-Norvaline and L- α -aminocaproic acid were prepared by enzymatic resolution of the corresponding racemic amino acids (11, 12). L-Glutamic acid, L-phenylalanine, and α -ketoglutaric acid were obtained from the Nutritional Biochemicals Corporation.

Phenylpyruvic acid was synthesized by the method of Herbst and Shemin (13). α -Ketoisovaleric acid was prepared by refluxing 2-phenyl-

* A preliminary report of this work has appeared (1).

4-isopropylidene-5-oxazolone (14) with 3 N hydrochloric acid for 5 hours. The keto acid was extracted with ether and purified by vacuum distillation (analysis, calculated, C 51.68, H 6.94; found, C 51.61, H 7.00).¹ The crystalline sodium salt was prepared by neutralization with sodium hydroxide followed by addition of an excess of acetone (Na, calculated 16.65; found, 16.55). Barium α -keto- γ -methiolbutyrate was prepared according to Cahill and Rudolph (15), and the barium removed quantitatively with sulfuric acid before use.

The d - α -keto- β -methylvaleric acid was prepared from D-alloisoleucine by the action of D-amino acid oxidase in the following manner: A mixture of 40 gm. of D-alloisoleucine dissolved in 1800 cc. of water and 3100 cc. of an aqueous extract of hog kidney acetone powder was adjusted to pH 8.2 with sodium hydroxide and stirred for 7 hours at 37°. A stream of oxygen was bubbled into the solution throughout incubation. The mixture was adjusted to pH 4.0 with hydrochloric acid and heated at 70° for 10 minutes with norit. The solution was filtered, concentrated *in vacuo* to 800 cc., and acidified to Congo red paper with concentrated hydrochloric acid. The keto acid was extracted with ether and purified by vacuum distillation. The crystalline sodium salt was prepared as described for sodium α -ketoisovalerate (Na, calculated, 15.12; found, 15.03). The sodium salt of d - α -keto- β -methylvaleric acid, which has the same configuration about the β -carbon as L-isoleucine, gave a specific optical rotation of +31.4° (0.7 per cent aqueous solution). The L isomer of the keto acid was prepared as the sodium salt in a similar manner from DL-isoleucine (Merck, lot No. 40761) (Na, calculated, 15.12; found, 15.18). The specific rotation of this salt was -32.6° (1 per cent aqueous solution), and the specific rotation of the 2,4-dinitrophenylhydrazone of the keto acid was -16.5° (2 per cent solution in ethanol). Greenstein, Levintow, Baker, and White (personal communication) observed rotations of -16.9° and +16.8°, respectively, for the 2,4-dinitrophenylhydrazones of the keto acids obtained from pure samples of D-isoleucine and D-alloisoleucine by the action of D-amino acid oxidase.

Oxalacetic acid was obtained by the method of Krampitz and Werkman (16). The α , γ -diketo acids, β , δ -diketocaproic acid and its δ -lactone, and dehydroacetic acid were synthesized and characterized (17, 18). β -Acetylacrylic acid was prepared according to Wolff (19) and purified by sublimation *in vacuo*. The β -keto acids were synthesized as the ethyl esters by the procedure of Fischer *et al.* (20) and saponified and extracted as described by Davies (21). The solutions of the sodium salts of the β -keto acids thus obtained were standardized by the aniline-citrate procedure (22). Ethyl acetoacetate, γ -ketovaleric acid, methyl ethyl ketone, methyl

¹ The microanalyses were performed by Mr. R. J. Koegel.

propyl ketone, and acetylacetone were Eastman products. Pyruvamide was synthesized according to Anker (23). The γ -methyl and γ -ethyl amides of L-glutamic acid and L-pyrrolidonecarboxylic acid were prepared according to Lichtenstein (24).

Carbamyl-L-glutamic acid was prepared as follows: 9 gm. of potassium cyanate (Eastman Kodak Company) were added to 100 cc. of a 1 M solution of L-glutamic acid neutralized to pH 6.8 with potassium hydroxide. After standing at room temperature (22–28°) for 48 hours, the mixture was cooled in ice and acidified to Congo red paper with concentrated hydrochloric acid. The product, which crystallized on scratching, was recrystallized from hot water. The melting point was 169° (corrected), which is somewhat higher than that given in the literature (25) (analysis, calculated, C 37.88, H 5.30, N 14.73; found, C 37.98, H 5.33, N 14.70). The γ -ethyl ester of L-glutamic acid was prepared according to Bergmann and Zervas (26). The pyridoxal phosphate was a gift from Dr. W. W. Umbreit.

Methods

Ammonia was determined as described by Greenstein and Leuthardt (27). α -Keto acids were determined by the method of Lu (28) and with crystalline lactic dehydrogenase and reduced diphosphopyridine nucleotide (DPNH₂) as previously described (10). The β -keto acids were determined by the procedure of Edson (22). The α,γ -diketo and β,δ -diketo acids were estimated by the spectrophotometric procedures previously reported (17, 18, 29). α -Ketoglutarate was determined in the presence of pyruvate, α -ketobutyrate, α -ketovalerate, or phenyl pyruvate in the following manner: A suitable aliquot of the reaction mixture was treated with an excess of crystalline lactic dehydrogenase and DPNH₂ in order to reduce the monocarboxylic α -keto acid. When reduction was complete (as judged by the absence of further decrease in the absorption of DPNH₂), 4 cc. of a 0.1 per cent solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid were added, and the mixture was allowed to stand for 15 minutes. Extractions with ethyl acetate and sodium carbonate were carried out according to Lu (28) and the colors compared with those of standards in a Coleman spectrophotometer at 600 m μ .

Glutamic acid and glutamine were determined by quantitative decarboxylation with *Clostridium welchii* (strain SR 12) suspensions according to Krebs (30). The reported values are based upon the quantity of carbon dioxide formed and are not corrected for ammonia formation as described by Krebs. Alanine, phenylalanine, and leucine were determined by microbiological methods with *Leuconostoc citrovorum* and *Leuconostoc mesenteroides* (31, 32).

Paper chromatograms were used for the detection and identification of

amino acids.² The following solvents were employed: phenol saturated with water in an atmosphere of hydrocyanic acid and ammonia; 77 per cent ethanol; formic acid (15 per cent), tertiary butanol (70 per cent), and water (15 per cent); and lutidine mixture (33). Authentic samples of the various amino acids and the experimental samples were run on the same set of paper strips.

Sprague-Dawley and Osborne-Mendel rats of both sexes, fed *ad libitum* and weighing 150 to 350 gm., were employed. Liver homogenates were prepared with 3 volumes of ice-cold distilled water in a small Waring blender and centrifuged at $20,000 \times g$ for 1 hour before use. The enzyme preparation used in these studies was prepared essentially according to Errera (6). The ethanol concentration of a homogenate of 300 gm. of liver was adjusted to 20 per cent by the addition of cold (-10°) ethanol. After standing at -8° for 12 hours, the precipitate was removed by centrifugation, and the supernatant adjusted to pH 5.3 with 0.2 M acetic acid. After standing at -8° for 2 to 3 hours, the precipitate was collected by centrifugation and suspended in 150 cc. of cold water. The suspension was stored at 0° for 2 to 3 hours, centrifuged, and the supernatant adjusted to pH 6.5 and an ethanol concentration of 35 per cent. The solution was allowed to stand at -8° for 12 hours and the precipitate was centrifuged and dissolved in 80 to 120 cc. of cold water, the insoluble residue being removed by centrifugation. The preparation, which contained about 1 mg. of protein nitrogen per cc., yielded no ammonia from glutamine in the absence of α -keto acids and was 30 to 50 times more active than the original homogenate (on the basis of protein nitrogen) in catalyzing the deamidation of glutamine in the presence of pyruvate. When stored at 0° , the preparation was stable for 4 or 5 days, followed by a rapid decrease in activity. Further purification, as described by Errera, led to a preparation which was about 20 per cent more active but which was otherwise similar to that described above.

Results

Effect of Keto Acids on Deamidation of Glutamine

The effect of a wide variety of keto acids on the deamidation of glutamine by rat liver extracts was studied under the conditions employed by Greenstein and Price (7). Only acids possessing an α -keto group produced significant acceleration of the deamidation of glutamine³ (Table I). The

² The authors are indebted to Dr. H. A. Sober for carrying out the chromatographic procedures.

³ The effect of various keto acids on the deamidation of asparagine was similar to that observed with glutamine in that only α -keto acids produced acceleration. Studies on the keto acid activation of asparagine deamidation will be reported at a later date.

mean value for eleven experiments with pyruvic acid was 8.88 μM of ammonia (range 7.67 to 10.4). This value and those given in Table I for phenylpyruvic and α -ketoisocaproic acids are in essential agreement with those of previous investigators (2-7). The following keto acids produced no significant increase in glutamine deamidation: α -ketoisovaleric, *d*- and *l*- α -keto- β -methylvaleric, the normal β -keto acids from acetoacetic to β -ketoundecylic, β , δ -diketocaproic, triacetic lactone, dehydroacetic, β -acetylacrylic, and γ -ketovaleric. Acetylacetone, acetone, methyl ethyl ketone, and methyl propyl ketone were also inactive. Pyruvamide did not increase the deamidation of glutamine nor was this compound enzymatically deamidated. The pH optimum for glutamine deamidation in

TABLE I
*Effect of Various Keto Acids on Deamidation of Glutamine in Liver Extracts**

Keto acid	Relative value	Keto acid	Relative value
Pyruvic	(100)	Oxalacetic	99
None	10	α , γ -Diketovaleric	91
α -Ketobutyric	98	α , γ -Diketocaproic	92
α -Ketovaleric	101	α , γ -Diketoheptanoic	87
α -Ketocaproic	100	α , γ -Diketooctanoic	95
α -Ketoheptanoic	93	α , γ -Diketnonanoic	73
α -Ketoocetanoic	80	α , γ -Diketocapric	72
α -Ketononanoic	51	α , γ -Diketoundecylic	43
α -Ketoisocaproic	67	α , γ -Diketo- δ -methylcaproic	41
Phenylpyruvic	64	α , γ -Diketo- ϵ -methylheptanoic	87
<i>p</i> -Hydroxyphenylpyruvic	64	α , γ -Diketo- γ -phenylbutyric	65
α -Keto- γ -methiolbutyric	64	α -Ketoglutaric	40

* The reaction mixtures contained initially keto acid (28 μM), glutamine (14 μM), 0.05 M veronal buffer at pH 7.1, and 1 cc. of extract in a volume of 4 cc.; incubated at 37° for 4 hours. The relative values are expressed as per cent of the value for pyruvic acid. Inactive keto acids (relative values 3 to 13) are listed in the text.

the presence of α -ketoglutaric and phenylpyruvic acids was found to be 8.0 to 8.5 (Fig. 1). The optima for glutamine deamidation with pyruvate and α -ketosiocaproate were reported to be pH 7.0 and 8.8, respectively (7).

From 90 to 95 per cent of the added β -keto acids was recovered at the end of the incubation period. However, in the case of the α , γ -diketo acids, only 5 to 50 per cent of the added amount was recovered, and corresponding quantities of pyruvic acid were formed. Although α , γ -diketo acids are rapidly hydrolyzed to pyruvic acid by liver extracts (17), Errera's preparation was found to possess only slight hydrolytic activity towards diketo acids. When α , γ -diketo acids were tested with this preparation, little glutamine deamidation occurred and no appreciable breakdown of diketo acid was observed (Table II). These results are com-

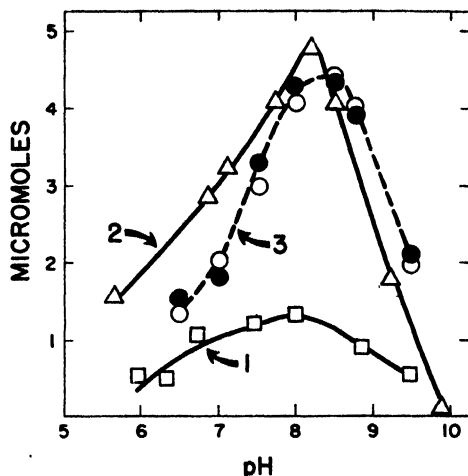


FIG. 1. pH-activity curves. Curves 1 and 2, deamidation of glutamine in the absence and presence of α -ketoglutaric acid, respectively; conditions as given in Table I. Curve 3, reaction mixtures contained glutamine ($10\ \mu\text{M}$), phenylpyruvic acid ($10\ \mu\text{M}$), 1 cc. of purified enzyme, and $0.05\ \text{M}$ veronal-acetate buffer, in a volume of 5 cc.; incubated for 2 hours. $\bullet$, disappearance of glutamine; $\circ$, formation of ammonia.

TABLE II

*Deamidation of Glutamine and Keto Acid Recovery with Purified Enzyme Preparation**

Keto acid added	NH ₃ formed	Keto acid found
	μM	μM
None	0	
Pyruvic (glutamine omitted)	0	27.4†
"	2.44	25.0†
β,δ -Diketocaproic	0.04	27.2
" (δ -lactone)	0	27.4
α,γ -Diketovaleric	0.63	25.2
α,γ -Diketocaproic	0.72	25.2
Oxalacetic	0.27	26.4
α -Ketoglutaric	1.20	27.6

* The reaction mixture contained initially $28\ \mu\text{M}$ of keto acid, $14\ \mu\text{M}$ of glutamine, veronal buffer at pH 7.1, and 1 cc. of enzyme in a volume of 4 cc. Incubated $\frac{1}{2}$ hour at 37° .

† Determined with crystalline lactic dehydrogenase and DPNH_2 .

patible with the findings obtained with primary rat hepatoma. Although pyruvate activated the deamidation of glutamine in extracts of hepatoma, α,γ -diketovalerate did not, and, since the neoplastic tissue was found to possess only a weak hydrolytic activity towards α,γ -diketo acids, it was

concluded that the diketo acid itself was inactive in the glutamine system (17). The effect of oxalacetic acid on glutamine deamidation by the purified enzyme preparation was about 10 per cent of the value obtained with the homogenate. Almost all of the added oxalacetate was recovered in these experiments, whereas none was recovered in experiments with liver extracts. Since β,δ -diketocaproic acid and its stable δ -lactone are metabolized to acetoacetate and acetate by liver homogenates (18, 34, 35), these keto acids were also tested with the purified preparation. No appreciable deamidation of glutamine occurred nor were these compounds hydrolyzed (Table II).

Transamination from Glutamine to α -Keto Acids with Purified Enzyme

It has been reported that 80 to 90 per cent of the pyruvate added to a digest of rat liver extract with glutamine could be recovered at the end of the incubation period. The procedure was based on the color intensity of an alkaline solution of the 2,4-dinitrophenylhydrazone (2-7). Employing the colorimetric procedure for the determination of keto acids, we have confirmed these findings for pyruvate and have obtained similar results with α -ketobutyrate and α -ketovalerate.

In several experiments with the purified enzyme preparation, pyruvate analyses were carried out with crystalline lactic dehydrogenase and reduced DPN. Although this enzyme system is not specific for pyruvate, it reduces certain keto acids such as oxalacetic and α -ketoglutaric acids at extremely low rates (10). Employing this technique for pyruvate determination, we observed that pyruvate disappeared in amounts approximately equimolar with ammonia formation. Furthermore, when the incubated samples were treated with an excess of lactic dehydrogenase and DPNH_2 , and subsequently with 2,4-dinitrophenylhydrazine, an alkali-soluble 2,4-dinitrophenylhydrazone was obtained which possessed an absorption curve similar to that of α -ketoglutaric acid with a maximum at 4200 Å. In addition, the formation of appreciable amounts of alanine in these mixtures was demonstrated by means of paper chromatography. Transamination was further demonstrated by quantitative determinations of glutamine, alanine, pyruvate, and α -ketoglutarate. Similar results were obtained with α -ketoisocaproic, α -ketovaleric, α -ketobutyric, and phenylpyruvic acids. The values for keto acid and glutamine disappearance and those for the formation of amino acid, α -ketoglutarate, and ammonia are given in Table III. Within experimental error, the disappearance of keto acid and glutamine in these experiments was associated with equimolar formation of α -ketoglutarate, amino acid, and ammonia. Good agreement between the values for ammonia formation and those for glutamine disappearance were obtained at various values of pH (Fig. 1).

The α -ketoglutarate formed was isolated as the 2,4-dinitrophenylhydrazone in the following manner: A mixture of 3 mM each of phenylpyruvic acid and glutamine, dissolved in 150 cc. of 0.1 M veronal buffer at pH 8.2, was incubated with 280 cc. of purified enzyme at 37° for 6 hours. The solution was deproteinized with trichloroacetic acid and treated with 650 mg. of 2,4-dinitrophenylhydrazine dissolved in 75 cc. of 2 N hydrochloric acid. After standing at 5° for 16 hours, the dinitrophenylhydrazone was filtered and recrystallized from hot water. The dried product weighed 486 mg. and had a melting point of 218° (uncorrected) (analysis, N,

TABLE III
*Transamination from Glutamine to α -Keto Acids**

Keto acid added	Incubation period	Keto acid disappearance	Amino acid formation	Glutamine disappearance	α -Ketoglutarate formation	Ammonia formation
	min.	μ M	μ M	μ M	μ M	μ M
Pyruvic.....	60	3.30	3.2†	3.20	3.32	3.18
"	120	5.00	4.2†	4.74	5.06	4.76
α -Ketoisocaproic.....	90		2.8‡	3.05		3.10
"	180		4.0‡	4.13		4.26
Phenylpyruvic.....	90	3.56	3.4§	3.80	3.15	3.20
"	180	5.10	4.7§	5.10	5.00	4.90
α -Ketobutyric.....	60	3.40		3.52		3.22
"	120	5.16		5.22	4.78	4.88
α -Ketovaleric.....	90	4.52		4.60		4.21
"	180	6.78		6.80	7.01	7.14

* The reaction mixtures contained initially 0.005 M keto acid, 0.0025 M L-glutamine, 0.05 M veronal-acetate buffer at pH 7.1, and 1 cc. of enzyme in a final volume of 4 cc.; incubated at 37°.

† Alanine.

‡ Leucine.

§ Phenylalanine.

calculated, 17.18; found, 17.20). The melting point of an authentic sample and a mixed melting point were 218–219° and 218°, respectively.

No deamidation of glutamine or transamination was observed with α -ketoisovaleric acid or *d*- and *l*- α -keto- β -methylvaleric acids with the purified enzyme preparation. Although deamidation of glutamine occurred with α -ketoglutarate, there was no change in the concentration of the keto acid. Studies with carbon-labeled α -ketoglutaric acid or glutamine would be necessary to demonstrate transamination in this system.

Studies with N¹⁵-Labeled Glutamine

Previous work has indicated that the increase in ammonia formation from glutamine due to the presence of pyruvate could be accounted for

by a corresponding decrease in the amide nitrogen group of glutamine, as determined by acid hydrolysis (2). However, in view of the possibility that the amide group of glutamine might serve as a precursor of the α -amino groups formed in this reaction, glutamine labeled with N^{15} in the amide group was employed. The ammonia formed in experiments with added pyruvate and phenyl pyruvate contained the same isotope concentration as did the amide group of the glutamine⁴ (Table IV). Within experimental error, all of the isotope was recovered as ammonia. These experiments therefore unequivocally demonstrate that the ammonia formed in the presence of added pyruvate or phenyl pyruvate arises from the amide group of glutamine and suggest that the α -amino group of the amino acid formed is derived from the amino group of glutamine.

TABLE IV
Experiments with Glutamine Containing N^{15} in Amide Group

Keto acid	Incubation period	NH ₃ found	N^{15} concentration
	<i>hrs.</i>	<i>mg.</i>	<i>atom per cent excess</i>
Pyruvic*.....	2	0.119	1.03
".....	4	0.170	1.04
Phenylpyruvic†.....	2	0.125	1.38
".....	4	0.174	1.40

* The reaction mixtures contained initially sodium pyruvate (20 μ M), glutamine (10 μ M), 0.05 M veronal buffer at pH 7.1, and 2 cc. of enzyme in a volume of 5 cc. The amide group of the glutamine contained 1.02 atom per cent excess of N^{15} .

† The reaction mixtures contained initially phenyl pyruvate (10 μ M), glutamine (10 μ M), veronal buffer at pH 8.1, and 2 cc. of enzyme in a volume of 5 cc. The amide group of glutamine contained 1.43 atom per cent excess of N^{15} .

Comparison of Glutamine with Glutamate in Transamination

The results thus far described could be explained on the basis of a two-step reaction involving an α -keto acid-catalyzed deamidation of glutamine yielding ammonia and glutamic acid, followed by transamination between glutamate and the α -keto acid. However, previous studies on transamination in pigeon muscle have shown that, although glutamic acid transaminates rapidly with pyruvate, the rates with other keto acids are relatively low (36). In contrast, the deamidation of glutamine, as well as the associated transamination reaction, occurs with a number of α -keto acids at rates of about the same order of magnitude. It was of importance, therefore, to compare transamination from glutamine with that from glutamic acid to various α -keto acids. The results of such a comparison

⁴ The authors wish to thank Dr. J. White for the isotope analyses.

indicate that transamination from glutamic acid to α -keto acids occurs at very low rates except in the case of pyruvic and α -ketobutyric acids (Table V). Under the same conditions, appreciable transamination takes place between glutamine and all of the α -keto acids studied except α -ketoisovaleric and α -keto- β -methylvaleric acids. The formation of alanine, α -aminobutyric acid, norvaline, leucine, tyrosine, methionine, and phenylalanine was demonstrated in the experiments described in Table V by paper chromatography. Under similar conditions, the formation of glutamic acid from various amino acids and α -ketoglutaric acid was found to occur at a very low rate except in the case of alanine and α -aminobutyric

TABLE V
*Comparison of Transamination with Glutamate and Glutamine**

Keto acid	Glutamate	Glutamine	
	Disappearance of glutamate	Disappearance of glutamine	NH ₃ formation
	μM	μM	μM
None	0.20†	0.05	0
Pyruvic	11.0	9.80	9.86
α -Ketobutyric	8.20	8.10	8.67
α -Ketovaleric	0.80†	9.10	9.95
α -Ketocaproic	0.56†	8.50	8.96
α -Ketoisocaproic	0.10†	6.40	6.78
Phenylpyruvic	1.09†	6.97	6.95
<i>p</i> -Hydroxyphenylpyruvic	1.11†	7.10	7.04
α -Keto- γ -methiolbutyric	0.50†	6.06	6.60

* The reaction mixtures contained initially 0.008 M keto acid, 0.004 M L-glutamine or L-glutamic acid, 0.05 M veronal-acetate buffer at pH 7.1, and 2 cc. of enzyme in a final volume of 5 cc. Incubated at 37° for 1 hour, except as indicated.

† Incubated 4 hours.

acid. When 10 μM each of α -ketoglutarate and L-amino acid were incubated under the conditions described in Table V, 0.5 to 1.5 μM of glutamate were formed in 4 hours with norvaline, α -aminocaproic acid, methionine, phenylalanine, tyrosine, and leucine, while the formation of 5 μM of glutamate was observed in 15 and 60 minutes with alanine and α -aminobutyric acid, respectively.

Study of the time-course of the glutamate-pyruvate and glutamine-pyruvate reactions indicates that the former reaction occurs more rapidly than the latter (Fig. 2). Similar results were obtained with α -ketobutyrate. On the other hand, in the case of phenyl pyruvate, as well as the remaining α -keto acids, the reaction was slower with glutamate. Although the curves for the disappearance of glutamine (as determined by de-

carboxylation with *C. welchii* suspensions) and for ammonia formation were about the same for the first half of the reaction, there was a relative increase in the ammonia formation towards the end of the reaction (Fig. 2). Since by the method employed for glutamine determination, glutamine cannot be distinguished from glutamate, these findings suggested that glutamic acid was formed by transamination between the α -keto-glutarate and amino acid formed. This hypothesis was supported by the finding of glutamic acid by paper chromatography and is compatible with

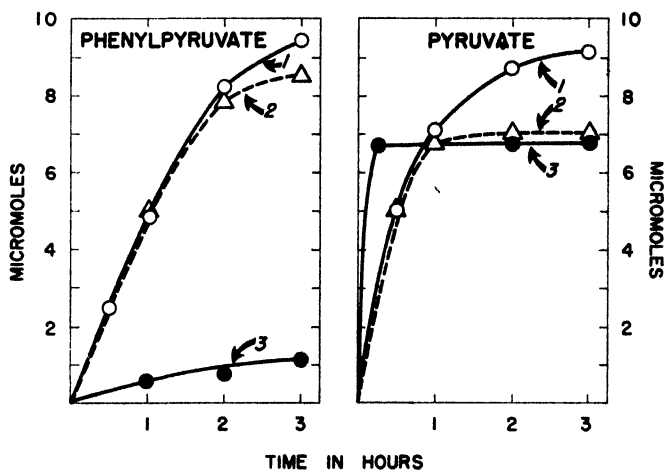


FIG. 2. Time-course of the reaction. Reaction mixtures contained initially keto acid ($20 \mu\text{M}$), glutamic acid or glutamine ($10 \mu\text{M}$), 0.05 M veronal buffer (pH 7.2), and 2 cc. of enzyme in a volume of 5 cc. Glutamine plus keto acid, Curve 1, ammonia formation; Curve 2, glutamine disappearance. Glutamate plus keto acid, Curve 3, glutamate disappearance.

the results of experiments on the formation of glutamate from α -keto-glutarate and various amino acids described above.

The low rate of transamination between glutamic acid and phenyl pyruvate (and α -ketovalerate) was not increased by addition of pyridoxal phosphate or ammonium chloride. Similar data were obtained when the glutamate was introduced slowly through a hypodermic needle over a 4 hour period by means of a Klett compensator connected to a tuberculin syringe.

Because impurities in glutamine isolated from natural sources have sometimes been responsible for anomalous results (37), synthetic L-glutamine was tested with several of the active α -keto acids. The results with the synthetic and isolated samples of glutamine were identical.

Effect of Other Glutamic Acid Derivatives

Acetylglutamic acid and isoglutamine were only as active as glutamic acid in transaminating with α -ketovalerate and phenyl pyruvate, although these compounds were hydrolyzed to completion during incubation with the enzyme preparation. The rates of hydrolysis of these compounds to glutamic acid were not increased by addition of α -keto acids. The effectiveness of other glutamic acid derivatives, *viz.* pyrrolidonecarboxylic acid, carbamylglutamic acid, and the γ -ethyl ester of glutamic acid in transaminating with α -ketovalerate and phenyl pyruvate, was of the same low order of magnitude as that of glutamate. Furthermore, the γ -ethyl ester of glutamic acid did not transaminate with pyruvate. The ester was not hydrolyzed in this system, as determined by the rate of decarboxylation by *C. welchii* suspensions.*

It was found that the γ -methylamide of glutamic acid was considerably more active than glutamic acid in transaminating with α -ketovaleric and phenylpyruvic acids, by measuring the rate of decrease of the concentration of the keto acids. In an experiment with 10 μ M of the methylamide, 20 μ M of α -ketovalerate, and 2 cc. of enzyme, in veronal buffer at pH 7.1, 6 μ M of keto acid disappeared in 3 hours. Under the same conditions, 9.5 μ M and 0.5 μ M of keto acid disappeared with glutamine and glutamic acid, respectively. The formation of a steam-volatile base in the experiments with the methylamide suggests that methylamine was formed. In contrast to the results with the methylamide, the γ -ethylamide of glutamic acid and glutathione were approximately as inactive as glutamic acid.

DISCUSSION

The present evidence suggests that α -keto acid-catalyzed deamidation of glutamine and transamination are associated. With the purified enzyme, neither reaction occurs in the absence of certain α -keto acids, and both deamidation and transamination occur at the same relative rates at various pH values. It has thus far not been possible to separate α -keto acid-catalyzed deamidation from transamination. It is noteworthy that α -ketoisovaleric and α -keto- β -methylvaleric acids are inactive in both reactions. The inactivity of these keto acids is of interest because, in contrast to the keto acids which were active in this system, these acids possess only 1 hydrogen atom on the β -carbon. Since glutamate reacted much more slowly than did glutamine, with all the active α -keto acids except pyruvic and α -ketobutyric acids, it appears unlikely that glutamic acid is an intermediate in this reaction. Thus, simple hydrolysis of gluta-

* *C. welchii* suspensions decarboxylate L-glutamic acid approximately 40 times faster than the γ -ethyl ester of L-glutamic acid. L-Pyrrolidonecarboxylic acid is not decarboxylated under these conditions.

mine to glutamic acid and ammonia is probably not the initial step of the reaction. It is possible that transamination and deamidation occur simultaneously, or that transamination precedes deamidation. The latter mechanism would involve the intermediate formation of α -ketoglutaramic acid and subsequent deamidation of this compound to yield α -ketoglutarate and ammonia.

It seems probable that the mechanism of the reaction between glutamine and α -keto acids is similar for all of the active α -keto acids. That transamination to pyruvate and α -ketobutyrate was more rapid with glutamate than with glutamine may be ascribed to the presence of glutamic-alanine transaminase in the purified enzyme preparation. The purified glutamic-alanine and glutamic-aspartic transaminases of muscle have been reported to be inactive with glutamine (38). It has been found in collaboration with Dr. H. P. Morris that livers of vitamin B₆-deficient rats and mice exhibit no decrease in the glutamine-pyruvate and glutamine-phenyl pyruvate deamidation reactions, although a considerable loss in glutamic-alanine transaminase activity was observed⁶ (cf. (39)). The system responsible for catalysis of the glutamine- α -keto acid reaction is probably not identical with either of the two classical transaminases.

It has been stated that glutamine cannot enter into transamination reactions unless previously split by hydrolytic enzymes (40). The present data indicate that glutamine may transaminate with certain α -keto acids without prior hydrolysis to glutamate. Elucidation of the mechanism of this reaction and its significance in the intermediary metabolism of amino acids must await the results of further investigation.

SUMMARY

1. Deamidation of glutamine by rat liver extracts and by a purified liver preparation was increased by a number of α -keto acids. Oxalacetate, α -ketoisovalerate, *d*- and *l*- α -keto- β -methylvalerate, pyruvamide, and acids possessing β -, γ -, or δ -keto groups were inactive. α , γ -Diketo acids were active only if hydrolyzed to pyruvic acid.

2. Transamination from glutamine to α -keto acids was demonstrated by isolation of the α -ketoglutaric acid formed as the 2,4-dinitrophenyl-hydrazone, identification and quantitative determination of the amino acid formed, and determinations of the disappearance of glutamine and α -keto acid.

3. With glutamine labeled with N¹⁵ in the amide group, and pyruvate or phenyl pyruvate, all of the isotope was recovered as ammonia, demonstrating unequivocally that the ammonia formed arises from the amide group of glutamine.

⁶ These experiments will be described in a subsequent report.

4. Transamination from glutamate to α -ketovalerate, α -ketocaproate, α -ketoisocaproate, phenyl pyruvate, *p*-hydroxyphenyl pyruvate, and α -keto- γ -methiolbutyrate occurred at a much slower rate than did transamination from glutamine to these keto acids. With pyruvate and α -ketobutyrate, transamination occurred more rapidly with glutamate, probably owing to the presence of glutamic-alanine transaminase in the enzyme preparation.

5. Other glutamic acid derivatives were no more active than glutamic acid itself in transamination with α -ketovalerate and phenyl pyruvate. However, the γ -methylamide of glutamic acid was appreciably more effective than glutamic acid.

6. The data indicate that transamination between glutamine and certain α -keto acids occurs in this system without prior hydrolysis of glutamine to glutamic acid and ammonia.

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THE EFFECT OF BERYLLIUM ON CERTAIN ENZYMES*

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Within recent years a number of reports have appeared demonstrating the toxicity of beryllium in humans and animals. Relatively little is known, however, about the mode of action of beryllium in the body. Klemperer, Miller, and Hill (1), as well as Grier, Hood, and Hoagland (2), have demonstrated an inhibitory action of beryllium on alkaline phosphatase and have suggested the possibility that this inhibition might form the basis of the toxicity of this metal. DuBois *et al.* (3), who have confirmed the beryllium inhibition of phosphatase, have also stated that adenosinetriphosphatase was inhibited by beryllium. In view of these reports, it became desirable to study a larger number of enzymes in order to ascertain whether or not the inhibitory action of beryllium is confined to phosphatase.

EXPERIMENTAL

Respiration—The effect of beryllium salts on the respiration of tissue slices was examined. Guinea pig liver slices were suspended in phosphate-buffered Ringer's solution containing 0.2 per cent glucose and the oxygen uptake was measured by the Warburg technique. Beryllium chloride, which was added from the side arm, had no effect on the oxygen consumption even in concentrations of 1 mm per liter. Under these conditions, beryllium phosphate precipitated; replacement of phosphate buffer by borate, however, did not alter the results, as is shown by the following experiment: Rat liver slices were incubated in Warburg vessels containing borate-buffered medium according to Feinstein and Stare (4), to which 0.2 per cent glucose was added. Oxygen consumption was measured, and, after 20 minutes, beryllium chloride was added from the side arm. The final concentration of beryllium was 10^{-3} mole per liter. During the initial period, the Q_{O_2} was -11.2 , and following the addition of beryllium it was -10.3 . The oxygen uptake closely paralleled that of a control vessel to which no beryllium was added.

Succinic Oxidase—Since impermeability of cellular membranes to beryllium might have been responsible for this apparent lack of sensitivity of

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respiratory enzymes to the metal, the effect of beryllium on the succinic oxidase system was tested. The enzyme preparation used was a fresh homogenate of guinea pig liver, and the oxidation of succinate was measured manometrically at 38°. Table I demonstrates that the succinic oxidase system was activated by beryllium. Included in Table I are data showing the activation of this enzyme system by aluminum, which was first described by Horecker, Stotz, and Hogness (5).¹

Glycolysis—The possible action of beryllium on the glycolytic reactions of the Embden-Meyerhof cycle was examined. Glycolysis was measured by the usual manometric technique, acetone powder prepared according to the directions of Green *et al.* (7) being used as enzyme preparation. This enzyme solution was extensively dialyzed. The detailed experimental conditions are given in Fig. 1.

TABLE I
Activation of Succinic Oxidase by Beryllium and Aluminum

	Additions		
	None	BeCl ₂ , 5×10^{-4} M per liter	Al, 5×10^{-4} M per liter
QO ₂	0.5	1.9	2.4

Each vessel contained 25 mg. of guinea pig liver homogenate, 6×10^{-3} mole of Na succinate per liter, and 1.6×10^{-5} mole of cytochrome *c* per liter. Phosphate buffer, pH 7.4.

Fig. 1 shows that, in the case of the complete system containing magnesium, there was no significant effect of beryllium on the enzymes involved in glycolysis.

Several of the enzymes of the glycolytic cycle depend for their action on the presence of magnesium. Enolase, as well as some enzymes which effect transfer of phosphate (8, 9), belongs to this group. The question whether beryllium can replace magnesium in activating these enzymes

¹ Because of the similarity of action of aluminum and beryllium on succinic oxidase and because of the suggestion of Ball and Cooper (6) that the activation of succinic oxidase by aluminum depended on inhibition of alkaline phosphatase, the effect of aluminum on alkaline phosphatase was tested. It was found that under the conditions described previously (1) no inhibition occurred in the presence of 10^{-3} mole per liter of aluminum chloride. 38 per cent inhibition was caused by a concentration of aluminum of 10^{-4} mole per liter. Maximal inhibition of 88 per cent occurred with 10^{-3} mole per liter of aluminum. The inhibition of alkaline phosphatase by aluminum resembles that caused by beryllium in that it can be counteracted by magnesium. The concentration of aluminum necessary for comparable inhibition, however, is nearly 100 times greater than that of beryllium.

was investigated. That this is not the case is demonstrated by the lower curves of Fig. 1 which show that the rate of glycolysis in the absence of magnesium was not increased by the addition of beryllium.

Adenosinetriphosphatase—The effect of beryllium on adenosinetriphosphatase was investigated. The enzyme was myosin prepared by the method of Greenstein and Edsall (10). Sodium adenosinetriphosphate was prepared from the commercial barium salt (Armour) by means of cation exchange as described by Polis and Meyerhof (11). All solutions were prepared with glass-redistilled water. The experimental conditions

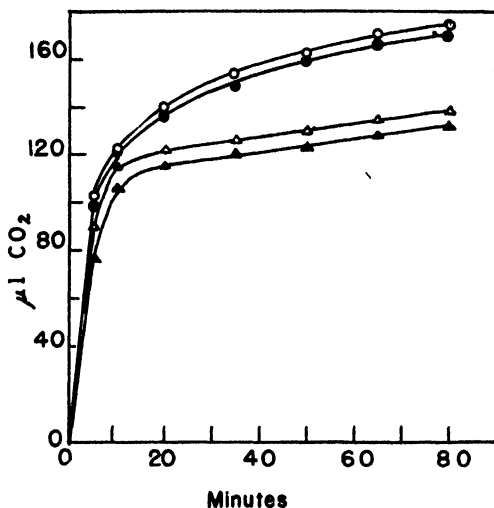


FIG. 1. Each vessel contained a dialyzed solution of 30 mg. of acetone powder, 0.04 mm of creatine, 0.01 mm of phosphate, pH 7.4, 0.01 mm of sodium hexose diphosphate, and adenosinetriphosphate in a concentration of 5×10^{-5} mole per liter. Additions, ○ magnesium 10^{-2} mole per liter; ● magnesium 10^{-2} mole per liter, beryllium 10^{-3} mole per liter; △ no additions; ▲ beryllium 10^{-3} mole per liter.

were as follows: 0.2 ml. of 0.1 N buffer was combined with the additions given in Table II, and the volume was brought to 0.55 ml. with water. Then 0.1 ml. of a solution containing 1.0 mg. of myosin in 0.5 M potassium chloride and 0.01 M potassium cyanide was added, and after temperature equilibration the reaction was initiated by quickly delivering 0.15 ml. of a 0.015 M solution of sodium adenosinetriphosphate. After 5 minutes the reaction was terminated by the addition of 0.1 ml. of 50 per cent trichloroacetic acid. The inorganic phosphate liberated was determined by the method of Fiske and Subbarow (12). Under these conditions, the amount of phosphate split was proportional to the concentration of the enzyme.

The data given in Table II demonstrate that in the presence of calcium there was no significant effect of beryllium on adenosinetriphosphatase. This is in disagreement with the data of DuBois *et al.* (3) who found inhibition of the enzyme in 8×10^{-4} M solutions of beryllium. When the experimental conditions of these authors were duplicated and the reaction time was extended to 15 minutes, gradual precipitation of myosin in the tubes containing beryllium occurred, with subsequent loss of enzyme activity. Precipitation of the enzyme apparently had not been observed by these authors because unpurified tissue homogenate had been used.

Table II demonstrates also that in the absence of calcium in borate solution at pH 9.2 beryllium activated adenosinetriphosphatase. This activation amounted to only 15 per cent, while that brought about by

TABLE II
Effect of Beryllium on Adenosinetriphosphatase

Buffers	pH	Additions	P split		
			No Be	Be, 0.1 mm per liter	Be, 1 mm per liter
			γ	γ	γ
Veronal.....	7.6	Ca, 3 mm per liter	21.5		22.3
"	7.6	None	14.5	14.0	
"	8.3	Ca, 3 mm per liter	16.5		16.6
"	8.3	None	8.2		7.3
Borate.....	9.2	Ca, 3 mm per liter	29.2		30.6
"	9.2	None	10.8	12.1	12.4

calcium was nearly 200 per cent. At greater hydrogen ion concentrations, no activation of adenosinetriphosphatase by beryllium was observed.

Table III demonstrates the lack of interaction of beryllium with a number of enzymes. All rates are expressed in per cent of the maximal rates observed in the presence of the required activators.

Carboxylase—Carboxylase was prepared from yeast and freed from co-enzyme and magnesium by repeated precipitation with ammoniacal ammonium sulfate according to the method of Green *et al.* (13). Its activity was determined by measuring carbon dioxide production from pyruvate under the conditions defined by these authors. It is seen that 10^{-4} mole per liter of beryllium did not inhibit the enzyme in the presence of co-carboxylase and magnesium, nor could it replace magnesium as an activator. When the concentration of beryllium was raised to 1 mm per liter, visible precipitation of the enzyme took place with complete loss of activity.

Arginase—The enzyme was prepared by extracting commercially defatted and dried liver powder (Viobin) with water and the extract was dialyzed against water. The activity of the enzyme was determined by the method of Hunter and Downs (14). Table III shows that arginase is neither inhibited by beryllium nor activated in the absence of manganese.

Carbonic Anhydrase—Meldrum and Roughton (15) have reported that beryllium had no effect on carbonic anhydrase. In their experiments, the liberation of carbon dioxide from bicarbonate was measured in the presence of phosphate buffer. Because of the extreme insolubility of beryllium phosphate, the question arose whether precipitation of beryllium might have accounted for the apparent insensitivity of this enzyme to beryllium. In order to avoid the presence of phosphate, we have em-

TABLE III
Effect of Beryllium on Various Enzymes

Enzyme	Addition	Per cent activity		Concentration of Be
		No Be	Be	
	<i>mole per l.</i>			<i>mole per l.</i>
Carboxylase	Thiamine phosphate, 6×10^{-4} , + MgCl_2 , 10^{-4}	100	103	10^{-4}
"	Thiamine phosphate, 6×10^{-4}	0	0	10^{-4}
Arginase	MnSO_4 , 3×10^{-4}	100	101	2.6×10^{-3}
"	None	86	85	2.6×10^{-3}
Carbonic anhydrase	"	100	106	10^{-3}
Uricase	"	100	100	10^{-3}

ployed the method of Philpot and Philpot (16), and measured the time of hydration of carbon dioxide in the absence of buffers. No visible precipitation occurred in the presence of beryllium. The data of Table II demonstrate that there was no inhibition of enzymatic activity by beryllium.

Uricase—This enzyme was prepared from acetone-dried pig liver by borate extraction and acetic acid precipitation as described previously (17). Its activity was measured gasometrically in borate buffer according to the procedure of Klemperer, Trimble, and Hastings (18). Again no inhibition by beryllium was noted.

DISCUSSION

In an attempt to correlate the toxic action of beryllium with its possible effect on certain biochemical reactions, it seemed advantageous first

to investigate systems in which the simultaneous action of a large number of enzymes is involved. For this reason respiring liver slices and glycolyzing muscle extracts were studied. These experiments revealed that beryllium has no effect on these systems.

Study of the succinate oxidase system revealed that beryllium in a concentration of 0.5 mm per liter produced a 4-fold increase of the rate of oxygen uptake. This activation appeared similar, although slightly smaller, than that which occurred in the presence of a similar concentration of aluminum.

The activation of succinic oxidase by aluminum has been explained variously by different authors. Ball and Cooper (6) maintained that it is due to inhibition of the destructive effect of phosphatase on a compound essential for the oxidation of succinate. If this were the case, the activation of succinic oxidase by beryllium could be explained by a similar mechanism, since beryllium inhibits alkaline phosphatase. On the other hand, Keilin and Hartree (19) and Bonner (20) have shown that activation of succinic oxidase can be brought about by a variety of colloidal agents or precipitates, including denatured globulin. They conclude, therefore, that the activation by aluminum is due to orientation of enzymes on the surface of the gelatinous precipitate. Since beryllium, under the conditions of the experiment, forms a gelatinous precipitate, similar to that of aluminum, it is likely that the activation of succinic oxidase by beryllium is also dependent on an alteration of the colloidal structure of the enzyme material. It appears that this activation is not specific and hardly of toxicological significance.

In the study of single enzymes attention was given to those which require divalent metals for activation. This was done because our earlier studies on alkaline phosphatase (1) had shown that its inhibition by beryllium could be counteracted by increasing concentrations of magnesium ions, an observation which suggested that the inhibition was based on competition of the divalent metals for the enzyme. The enzymes studied were adenosinetriphosphatase, arginase, and carboxylase. None of these enzymes was inhibited by beryllium unless its concentration was raised to the point at which precipitation of the enzyme resulted. There was slight activation of adenosinetriphosphatase by beryllium when magnesium was absent. Under such conditions, slight activation of phosphoglucomutase by beryllium had been reported by Stickland (21). Beryllium, however, could not replace other divalent metals in their activating function on enzymes of the glycolytic system, arginase, or carboxylase.

The effect of beryllium on carbonic anhydrase was studied because this

enzyme contains zinc and the possibility of competition with this metal seemed possible. No such phenomenon, however, could be demonstrated.

If the inhibition of alkaline phosphatase depended on the acidic property of beryllium hydroxide, it would be conceivable that interaction between this metal and a protein becomes apparent only at low hydrogen ion concentrations favoring the acidic dissociation of the hydroxide. For this reason the effect of beryllium on uricase, which shows maximal activity at pH 9.2, was examined. No inhibition was shown in this case.

The fact that a variety of enzymes was not inhibited even by high concentrations of beryllium while alkaline phosphatase had been shown to be sensitive to concentrations of beryllium as low as 10^{-6} M demonstrates a considerable specificity of beryllium for this enzyme, which lends support to the concept that interference with phosphatase action is related to the toxicity of beryllium.

SUMMARY

The effect of beryllium on various enzymes was tested.

Beryllium had no effect on the respiration of liver slices.

Succinic oxidase was activated by beryllium. This activation was similar to that which is caused by aluminum.

Beryllium does not inhibit glycolysis in muscle extracts; it cannot replace magnesium as an activator of this reaction.

Adenosinetriphosphatase, carboxylase, arginase, carbonic anhydrase, and uricase are not inhibited by beryllium.

In the absence of calcium there was slight activation of adenosinetriphosphatase by beryllium. Carboxylase and arginase are not activated by beryllium in the absence of other activating metals.

The previously reported inhibitory action of beryllium on alkaline phosphatase appears to be specific.

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THE EFFECT OF FATTY ACIDS ON THE GROWTH OF STRAINS OF *LACTOBACILLUS BIFIDUS**

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The explanation for the preponderance of *Lactobacillus bifidus* in the intestine of infants being fed human milk has been sought in a number of investigations. Such studies have been hampered by difficulties encountered in isolating strains of the organism. It has been a common experience that organisms that are bifid on primary isolation soon change, presumably by either selection or mutation, to a rod-like morphology (1).¹ All such straight rod strains isolated in this laboratory could be grown on a semisynthetic medium if supplemented with sources of unsaturated fatty acids. Several strains required an additional supplement of desoxyriboside (2). Human milk digested with pancreatin did not prove to be nutritionally superior to similar preparations of cow's milk when added as a supplement to the basal medium. It was observed, however, that high concentrations of the digested cow's milk but not of human milk inhibited growth of the straight rod strains. The toxic factor was found in the fatty acid fraction of cow's milk. The fatty acids separated from digested human milk proved to be of equal toxicity. It was soon found that there were substances present in both milks that neutralized the toxicity of the fatty acids liberated by digestion (2).

Recently methods have been developed that permit the isolation and maintenance of strains of *L. bifidus* without loss of the bifid morphology.¹ The study of inhibition of microbial growth by fatty acids and of factors protective against such inhibition were extended to include both the straight rod and bifid strains of *L. bifidus*.

EXPERIMENTAL

Media and Strains of L. bifidus—The four bifid and the one straight rod strains used in the present study were isolated from the stools of

* This investigation was supported by a grant from Wyeth Incorporated, Philadelphia.

¹ Norris, R. F., Flanders, T., Tomarelli, R. M., and György, P., manuscript in preparation.

breast-fed infants in the manner described in a previous publication (2). The composition of the basal medium was that presented earlier (2) with 70 gm. of lactose per liter of double strength medium substituted for the glucose. The addition of 0.1 per cent Tween 80 made the medium nutritionally complete for the straight rod strain. The bifid strains grew well on the basal medium alone, but slightly better growth was obtained when 1 mg. of pancreatin, 10 mg. of ascorbic acid, and 0.1 γ of vitamin B₁₂ were added to 10 cc. of the culture medium; a Seitz-filtered solution of the last two supplements was added aseptically to the previously autoclaved medium. This latter medium is designated the bifid medium. All supplements, including the fatty acids, were adjusted to pH 6.5 before addition to the assay tubes. In one series of experiments sodium citrate was

TABLE I

Effect of Digested Human and Cow's Milk on Growth of Strains of L. bifidus
Bifid medium, 40 hours anaerobic-CO₂ incubation.

Digested milk* per tube	0.1 N acid			
	Human		Cow	
	Rod	Bifid	Rod	Bifid
cc.	cc.	cc.	cc.	cc.
None	2.6†	19.5	2.6	19.5
1	16.0	0	5.9	0
0.1	12.7	0	12.5	0
0.01	15.5	0	16.0	0
0.001		17.8		18.9

* 72 hours digestion with 0.4 per cent pancreatin and 0.1 per cent bile salt.

† Slight growth produced by unsaturated fatty acid present in pancreatin of the medium.

used in place of sodium acetate. To each 5 cc. of this medium were added 100 mg. of dicalcium phosphate, 10 mg. of manganese sulfate, and 5 mg. of magnesium sulfate to compensate for the binding of bivalent cations by citrate (2, 3). The bifid strains were cultured in an anaerobic atmosphere containing 10 per cent carbon dioxide. The straight rod strain grew well under either aerobic or anaerobic conditions. All strains were incubated at 37°. The extent of growth of the organisms was determined by titrimetric assay, the details being those previously reported (2).

Toxicity of Digested Milk—Previous experiments (2) have shown that pancreatin digestion of whole milk produces digests that are toxic to the straight rod strain of *L. bifidus*. Cow's milk in most experiments yielded a more toxic digest than did human milk. The bifid strains proved to be much more sensitive to the toxicity of digested milk than did the rods. The data of a typical experiment, Table I, show that a 100 times more

digested milk was required for the inhibition of the straight rod strain than of the bifid strain. The toxic factor was found associated with the fatty acid fraction; *i.e.*, concentration was achieved by extraction of the acidified milk digest with equal parts of ethyl and petroleum ether, followed by a dilute alkali extraction of the ether extract.

The presence of factors in both cow's and human milk that decrease the toxicity of the fatty acids is demonstrated by the experiment in Table II. Ether extracts of milk samples that were digested only for a short time were more toxic than the original, unextracted digests. The addition of undigested milk completely neutralized the inhibition of the toxic extract. The effect of albumin in reversing the inhibitory action of oleic acid on the growth of the tubercle bacilli (4) suggested that the lactalbumin of

TABLE II
Toxic and Protective Factors in Digested Milk

Bifid strain, 40 hours anaerobic-CO₂ incubation.

0.5 cc. milk supplement per tube	0.1 N acid	
	Human	Cow
	cc.	cc.
1. None	24.2	24.2
2. Undigested	24.5	24.3
3. 1½ hrs. pancreatin-digested	6.6	19.4
4. Fatty acid fraction of (3)	2.0	9.9
5. (4) + (2)	23.9	24.0
6. (4) + dialyzed whey*	23.8	
7. (4) + 50 mg. bovine albumin†	25.0	24.5

* Calculated to contain 25 mg. of albumin.

† Added aseptically to autoclaved medium.

milk might be the protective factor. Dialyzed whey had protective activity, as did bovine serum albumin (Table II); casein was relatively inactive. Exhaustive alcohol extraction, heating with acid or alkali, and tryptic digestion all decreased the protective activity of the dialyzed whey.

Growth Inhibition by Fatty Acids—The effect of pure fatty acids on the growth of the straight rod and bifid strains of *L. bifidus* is summarized in Table III. Both strains give evidence of a similar sensitivity to the toxicity of the saturated fatty acids: lauric and myristic were the most toxic with capric and palmitic less toxic. In addition to the fatty acids listed in Table III, propionic, butyric, valeric, isovaleric, and isocaproic were tested and found non-inhibitory. In all of the experiments in which acetate medium was used, the unsaturated fatty acids, oleic and linoleic, proved toxic only to the bifid strains. The straight rod strain, which

requires unsaturated fatty acid for growth, was not inhibited by concentrations of oleic acid as great as 10 mg. per tube. In contrast, the bifid strains were definitely inhibited by concentrations of less than 100 γ per tube. Linoleic acid was more toxic than oleic acid, presumably because of the greater degree of unsaturation (5). Only the free fatty acids (soaps) were toxic to the lactobacilli. Ethyl laurate was not growth-inhibitory at a concentration of 1 mg. per tube for either the straight rod or the bifid strain. As demonstrated in the data of Table V, compounds with an esterified unsaturated fatty acid component, *i.e.* methyl oleate, methyl linoleate, Tween 80 (oleate ester of the polyoxyethylene derivative of sorbitan), and the phosphatide, lipositol,² were not toxic to the bifid strain.

TABLE III

Growth Inhibition of L. bifidus by Fatty Acids

Basal medium, 40 hours anaerobic-CO₂ incubation.

Fatty acid	Micrograms per tube* for 50 per cent inhibition			
	Rod†	Bifid 1	Bifid 2	Bifid 3
Caproic.....	No inhibition	No inhibition	No inhibition	No inhibition
Caprylic.....	" "	" "	" "	" "
Capric.....	1750	400	280	400
Lauric.....	350	150	150	170
Myristic.....	800	150	150	250
Palmitic.....	2150	350	180	380
Stearic.....	No inhibition	No inhibition	No inhibition	No inhibition
Oleic.....	" "	60	50	120
Linoleic.....	" "	20	20	40

* 2.5 mg. per tube, highest concentration tested.

† 1 mg. of oleic acid added for growth of the straight rod strain.

Firmly adsorbed unsaturated fatty acid, such as is present in the bovine serum albumin fraction or in dialyzed whey, was also non-toxic. The nutritional requirement of the straight rod strain for unsaturated fatty acid was supplied by either the free, esterified, or adsorbed form of the fatty acid. When citrate medium was used, both bifid and straight strains required a source of unsaturated fatty acid for growth, and for both strains free oleic acid was inhibitory. With Tween 80, or better with both Tween 80 and oleic acid, growth was supported (Table IV).

Protective Factors against Fatty Acid Toxicity—The inhibition of the

² The sample of lipositol was kindly supplied by Dr. F. W. Bernhart, Nutritional Division, Wyeth Incorporated, who has informed us in a personal communication of the growth stimulation of the phosphatide for lactobacilli requiring unsaturated fatty acid.

TABLE IV

Unsaturated Fatty Acid Requirement and Toxicity for Strains of L. bifidus on Acetate and Citrate Media

Bifid medium,* 40 hours anaerobic-CO₂ incubation.

Supplements	Acid production, 0.1 N			
	Bifid		Straight	
	Acetate	Citrate	Acetate	Citrate
	cc.	cc.	cc.	cc.
None.....	19.1	0	1.3	3.5
500 γ oleic acid.....	0.2	0	10.5	0.6
100 " " ".....	19.1	2.3	3.0	0
5 mg. Tween 80.....	20.2	10.1	6.9	0.6
5 " " 80 + 500 γ oleic acid.....	22.3	15.2	16.3	10.5
5 " " 80 + 100 " " ".....	20.6	9.0	9.8	10.9

* No pancreatin was used in this experiment.

TABLE V

Reversal of Fatty Acid Toxicity for Strains of L. bifidus

Bifid medium, 40 hours anaerobic-CO₂ incubation.

Supplement	0.1 N acid			
	Rod		Bifid	
	No acid	1000 γ lauric	No acid	250 γ oleic
	cc.	cc.	cc.	cc.
None.....	0.2	0	21.0	0.2
1 mg. oleic acid.....	13.9	0		
1 " methyl oleate.....	14.2		21.4	
1 " " linoleate.....	13.7		20.4	
1 " Tween 80.....	14.5	14.1	21.0	21.8
1 " " 60.....	0.3		20.8	18.9
1 " " 60 + 1 mg. oleic acid.....	14.0	11.03		
1 " lipositol.....	13.8	14.3	20.5	19.7
50 mg. bovine albumin*.....	15.0	14.4	18.5	18.9
25 " human lactalbumin†.....	14.7	15.0	20.2	20.9
50 " acacia.....	0.4		20.2	21.0
50 " " + 1 mg. oleic acid.....	13.9	14.2		

* Bovine serum albumin, Armour.

† Dialysed whey.

growth of a number of lactobacilli by high concentrations of oleic acid is overcome by the further addition of surface-active compounds (5, 6). The data of Table V demonstrate that the emulsifying agents Tween 80,

Tween 60, and lipositol neutralized the toxicity of lauric acid for the straight rod strain and protected the bifid strain from both oleic and lauric acid inhibition. Suspensions of acacia, dialyzed whey, and bovine serum albumin were also effective in the protection of both strains from fatty acid toxicity. The amounts of Tween 80 required to reverse fatty acid toxicity increased with increased concentration of the toxic substance; the ratio required for the reversal was not constant.

DISCUSSION

The microbiological population of the bowel of the breast-fed infant is composed almost entirely of lactobacilli, approximately 20 per cent of the organisms having a bifid morphology. Straight rod and bifid strains of the intestinal organism were found to be inhibited by the fatty acids liberated from whole milk by pancreatin digestion. This is in accordance with the well known bacteriostatic effect of long chain fatty acids on Gram-positive organisms (7). While both strain types exhibited a similar sensitivity to the C_{12} and C_{14} saturated fatty acids, a marked difference was found with respect to the C_{18} unsaturated fatty acids. When an acetate medium was used, the straight rod strain required approximately 1000 γ for maximal growth, an amount that far exceeded that necessary for complete inhibition of the growth of the bifid strains. High concentrations of oleic acid have been shown to be toxic to other lactobacilli that require low concentrations of the unsaturated fatty acid for growth (6). That the bifid strain requires unsaturated fatty acid for growth on the citrate medium recalls the fact that acetate has a nutritional as well as a buffer effect in the medium. Guirard *et al.* (8) observed that for certain lactic acid bacteria oleic acid could substitute, at least in part, for acetate. From the point of view of our experiments acetate could replace oleate for the bifid but not for the straight strain.

The bacteriostatic effect of long chain fatty acids is generally accepted as a physicochemical phenomenon (7, 9), although the evidence available does not exclude a specific metabolic effect. According to the physicochemical theory, the binding of the fatty acid to components in the wall of the bacterial cell blocks the entrance of essential nutrients or produces changes in the permeability of the cell membrane which result in the loss of nutrients into the medium. Regardless of the mechanism of toxicity the protective effect of serum albumin and of acacia against the growth inhibition of the bifidus strains by fatty acids may be explained by the removal of the toxic lipide from the medium by adsorption on the protective colloidal substance. Surface-active compounds such as Tween 80, Tween 60, and lipositol similarly detoxify by forming molecular associations with the toxic fatty acid.

It is difficult to explain the predominance of *L. bifidus* only in the intestine of the breast-fed infant on the basis of the fatty acid composition of human and cow's milk. The contents of the toxic saturated fatty acids are approximately equal; the lauric and myristic acid content of the fatty acids of human milk is 4.8 and 6.6 per cent, and of cow's milk 1.7 and 9.3 per cent, respectively (10, 11). Since human milk fatty acids are more unsaturated than in cow's milk, 55 and 35 per cent respectively, it would be expected that human milk would be more antagonistic to the sensitive bifid strains of *L. bifidus*. The toxicity of the contents of the infant's intestine would be dependent on the ratio of toxic fatty acids to neutralizing substances. It may be that the amount and type of protective factors are the determining factors for predominance of *L. bifidus* in the intestine of the breast-fed infant.

SUMMARY

The growth of straight rod and bifid strains of *Lactobacillus bifidus*, isolated from the stools of breast-fed infants, was inhibited by the fatty acids liberated from human and cow's milk by pancreatin digestion. Lauric and myristic acids were the most toxic of the saturated fatty acids tested and inhibited the growth of both the straight rod and bifid strains. With a medium containing sodium acetate the unsaturated fatty acids, oleic and linoleic, were markedly inhibitory to the growth of the bifid strains but not of the straight rod strain; only the straight rod strain required unsaturated fatty acid for growth. When sodium citrate was substituted for the sodium acetate buffer, both bifid and straight rod strains required a source of unsaturated fatty acid for growth and for both strains free oleic acid was inhibitory. Factors which alleviate the toxicity of fatty acids were demonstrated to be present in both human and cow's milk. Bovine serum albumin, dialyzed human whey, and acacia suspension were protective against the inhibitory action of the unsaturated fatty acids to the bifid strains, as well as to lauric acid toxicity to both the straight rod and bifid strains. A similar reversal of fatty acid toxicity was afforded by surface-active compounds, such as Tween 80, Tween 60, and lipositol.

The authors acknowledge with appreciation the technical assistance of Evelyn Poster, Annabel Avery, Thelma Flanders, and Fern Drexel.

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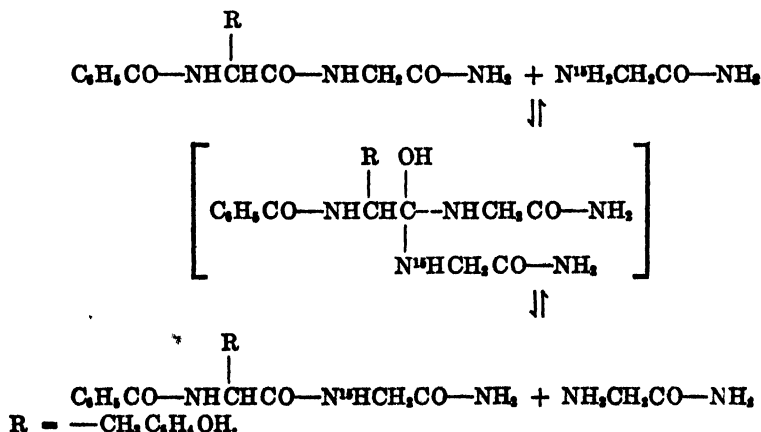
CATALYSIS OF TRANSPEPTIDATION REACTIONS BY CHYMOTRYPSIN*

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In earlier communications from this laboratory (1, 2) it was shown that intracellular proteinases such as papain, under suitable conditions, catalyze replacement reactions involving amide bonds (transamidation reactions). The present report deals with the catalysis of similar reactions by crystalline pancreatic chymotrypsin. The data in Table I describe the result of an experiment in which benzoyl-L-tyrosylglycinamide (BTGA), a typical synthetic substrate for chymotrypsin (3), was incubated with the enzyme in the presence of glycine which contained N^{15} in the glycine N. The enzymatic reaction was allowed to proceed at pH 7.9 until a fraction of the substrate had undergone hydrolysis, and the residual BTGA was isolated, purified, and analyzed for its isotope content. It will be seen from Table I that an appreciable quantity of N^{15} was found; the isotope concentration of 1.93 atom per cent excess N^{15} indicates that at least 17 per cent of the residual substrate had participated in a replacement reaction, leading to the incorporation of N^{15} . The reaction may be formulated as in the accompanying diagram, the postulated intermediate being enclosed in brackets.



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The catalysis of a replacement reaction by a crystalline proteinase supports the view, expressed earlier (1, 2), that enzymes of this group may act as catalysts in transamidation (or transpeptidation) reactions, as well as in hydrolytic or synthetic reactions involving peptide bonds. In the reaction represented, the replacement involves one of the components of a bond linking 2 amino acid residues and therefore may be termed a trans-

TABLE I
Catalysis of Transpeptidation by Chymotrypsin

Concentration of crystalline chymotrypsin (Armour), 0.072 mg. of protein N per cc. of test solution; 0.3 cc. of methanol per cc. of test solution; 0.05 M phosphate buffer, pH 7.9; temperature, 25°.

Substrate	Replacement agent	Hydrolysis of substrate		Residual substrate*		
				N content	N ¹⁵ concentration	
					Com- pound	Peptide N†
		min.	per cent	per cent	atom per cent excess	atom per cent excess
Benzoyl-L-tyrosylgly- cinamide (0.05 M)	N ¹⁵ H ₂ CH ₂ CONH ₂ ‡ (0.05 M)	78	42§	12.46	1.930	5.79
		120	0§	12.49	0.013	0.039
Benzoyl-L-tyrosin- amide (0.025 M)	N ¹⁵ H ₄ NO ₃ ¶ (0.05 M)	120	28**	9.68	0.015	0.030
		120	0**	9.90	0.005	0.010

* Isolated in the manner described in the experimental section.

† "Peptide N" refers to the nitrogen atom participating in the bond broken by the enzyme.

‡ This compound was prepared from isotopic glycine ethyl ester hydrochloride by treatment with ammonia in methanol (4). N¹⁵ concentration of glycine N, 33.87 atom per cent excess. Calculated for isotopic C₂H₄ON₂·HCl, 25.6 per cent nitrogen; found, 25.3 per cent.

§ Determined by formol titration as described by MacAllister *et al.* (5).

|| No enzyme present.

¶ Eastman preparation containing 63.8 atom per cent excess N¹⁵ in ammonium N.

** Determined by measurement of ammonia liberation in Conway vessels (2).

peptidation reaction. It was of interest to examine the extent to which chymotrypsin would catalyze a transamidation reaction in which benzoyl-L-tyrosinamide (BTA) was the substrate and isotopic ammonia (in the form of N¹⁵H₄NO₃) served as the replacement agent. It will be noted from Table I that, under the same conditions which led to appreciable transpeptidation with BTGA, the extent of isotope incorporation was very slight.

The difference in the results obtained in the two experiments points to the fact that, in the consideration of replacement reactions catalyzed by proteolytic enzymes, a specificity factor must be added to the well known structural requirements for the action of these enzymes in hydrolytic reactions (6-8). As suggested previously (1, 2), the catalysis of hydrolysis and of transamidation (or transpeptidation) appears to involve the activation of a carbonyl addition reaction; however, a series of replacement agents (*e.g.*, NH_3 , $\text{NH}_2\text{R}'$, $\text{NH}_2\text{R}''$, etc.) may be expected to exhibit differences in the rates of their reaction with the activated carbonyl group. At a given pH, for example, the relative concentrations of the conjugate bases may be different, depending on the pK value of the respective cations. Such quantitative differences in the extent of dissociation may, in part, be responsible for the difference found between the rates of the replacement reactions involving ammonia in the case of BTA and glycynamide in the case of BTGA. On the basis of these considerations it may be suggested that the absence of appreciable isotope incorporation in the experiment with BTA arises from an unfavorable relationship between the rates of transamidation and of hydrolysis, thus preventing the accumulation of isotopic BTA.

It was shown previously (2) that cysteine-activated papain can catalyze the transamidation reaction involving the replacement of a $-\text{NH}_2$ group of an acylamino acid amide by the $-\text{NHOH}$ group of hydroxylamine. The resulting hydroxamic acids may be estimated colorimetrically by converting them to their characteristic red ferric complexes. More recent experiments with cysteine-activated ficin have given results completely analogous to those obtained with papain (Table II). It will be noted that the extent of accumulation of hydroxamic acid at pH 7.2 is much greater than at pH values near 5, at which ficin exhibits optimal catalytic activity in the hydrolytic reaction.

Similar transamidation experiments were conducted with crystalline chymotrypsin; BTA, BTGA, and glycyl-L-phenylalaninamide (GPA) were used as substrates, and hydroxylamine served as the replacement agent. Control experiments showed that, under the conditions of these tests, hydroxylamine was without appreciable effect on the activity of chymotrypsin in promoting the hydrolysis of its substrates. It will be seen from Table II that, at pH 7.1 to 7.5, only small quantities of the expected hydroxamic acids can be demonstrated. These results are concordant with the view that the hydroxamic acids are rapidly hydrolyzed by chymotrypsin at these pH values. A recent report (10) has described the use of benzoyl-L-phenylalanylhydroxamic acid as a substrate for chymotrypsin.

Earlier studies from this laboratory (11) have demonstrated the presence, in extracts of swine kidney, of an intracellular proteinase which has a

TABLE II

Catalysis of Hydroxamic Acid Formation by Proteinases

Substrate concentration, except where otherwise stated, 0.05 M; concentration of $\text{NH}_2\text{OH}\cdot\text{HCl}$ (adjusted to pH 6 with NaOH), 0.05 M; 0.05 M citrate-phosphate buffers.

Enzyme	Substrate	pH	Time	Ammonia liberation*	Hydrolysis of peptide bond†	Hydroxamic acid present‡
			min.	μM per cc.	μM per cc.	μM per cc.
Chymotrypsin§	Benzoyl-L-tyrosylglycinamide	7.5	30		8.5	0.2
			60		15.0	0.3
	Benzoyl-L-tyrosinamide (0.025 M)	7.6	60	6.6		0.04
			180	13.9		0.08
	Glycyl-L-phenylalaninamide	7.1	60	10.2		0.1
Trypsin¶	Benzoyl-L-argininamide	7.5	300	22.9		0.13
			60	8.6		0.14
Ficin**	"	5.4	300	26.5		0.16
			30	20.4		1.4
Beef spleen cathepsin††	Glycyl-L-phenylalaninamide	7.2	30	19.6		4.7
		5.2	100	23.4		0.8
		6.6	100	17.6		1.8
	Benzoyl-L-argininamide	6.5††	100	0		0
		5.0	100	12.2		0.3
		6.5	100	8.0		0.5
		6.5††	100	0.6		0.01

* Determined by measurement of ammonia liberation in Conway vessels (2).

† Determined by formol titration as described by MacAllister *et al.* (5).

‡ Determined as described previously (2).

§ Armour preparation. Enzyme concentration in experiments with benzoyl-L-tyrosylglycinamide and with benzoyl-L-tyrosinamide, 0.07 mg. of protein N per cc. of test solution; with glycyl-L-phenylalaninamide, 0.60 mg. of protein N per cc. of test solution. The experiments were conducted at 25°.

|| 0.3 cc. of methanol present per cc. of test solution.

¶ Armour preparation. Enzyme concentration, 0.016 mg. of protein N per cc. of test solution. The experiments were conducted at 25°.

** Merck preparation. Enzyme concentration, 0.32 mg. of protein N per cc. of test solution. The experiments were conducted at 38° in the presence of 0.05 M cysteine.

†† This material was prepared as described previously (9). Enzyme concentration in experiments with glycyl-L-phenylalaninamide, 0.23 mg. of protein N per cc. of test solution; with benzoyl-L-argininamide, 0.7 mg. of protein N per cc. of test solution. The experiments were conducted at 38° in the presence of 0.05 M cysteine. ¹⁴

‡‡ No cysteine present.

specificity similar to that of pancreatic chymotrypsin. This enzyme (cathepsin C) has been found in other animal tissues, notably beef spleen, from

which a highly purified preparation has been obtained.¹ In its action on GPA, cathepsin C exhibits optimal hydrolytic activity near pH 5, and the addition of cysteine results in an activation of the enzyme. Experiments were performed to determine whether cathepsin C could catalyze hydroxamic acid formation in the presence of hydroxylamine when GPA was used as the substrate. The data in Table II show this to be the case and demonstrate further that the transamidation reaction is favored by a shift in pH from 5.2 to 6.6. Since the cathepsin preparation employed for these studies also contained the trypsin-like intracellular proteinase, cathepsin B (previously termed cathepsin II (12)), which acts on benzoyl-L-argininamide (BAA), comparative data are presented in Table II on the catalysis of hydroxamic acid formation from GPA and BAA by this enzyme preparation. It is of some interest that, as in the case of the cysteine-activated papain (2), hydroxylamine is a potent inhibitor of cathepsins B and C and of ficin, which are also activated by cysteine. On the other hand, hydroxylamine does not inhibit appreciably the hydrolytic activity of chymotrypsin or of trypsin, neither of which is activated by cysteine.

When crystalline pancreatic trypsin was used as the enzyme in experiments in which BAA was incubated with hydroxylamine at pH 7.5, no appreciable hydroxamic acid accumulation could be demonstrated (Table II). In view of the results obtained with chymotrypsin, it appears likely that the relative rates of transamidation and of hydrolysis of the expected hydroxamic acid are such as to favor the latter process.

EXPERIMENTAL

Isolation of Benzoyl-L-tyrosylglycinamide (BTGA) in Transpeptidation Experiments—427 mg. of BTGA were dissolved in 7.5 cc. of methanol, and 138 mg. of isotopic glycine hydrochloride were added. After the introduction of alkali, buffer, and enzyme, the mixture was adjusted to 25 cc. with water and incubated at 25° for 78 minutes. Aliquots were removed at the start and end of the reaction for formol titration and determination of pH. The remainder of the solution (23 cc.) was poured into 250 cc. of absolute alcohol and chilled for 2 hours, and the resulting precipitate was filtered with the aid of analytical Filter-Cel. The filtrate was concentrated to dryness under reduced pressure, and the residue was taken up in 6 cc. of hot water. The extract was clarified by filtration, adjusted to pH 6, and chilled overnight. The crystals which separated were filtered and recrystallized three times from 5 cc. portions of hot

¹ Tallan, H. H., Jones, M. E., and Fruton, J. S., unpublished experiments.

water. Yield, 72 mg. Isotope analysis of the material obtained after three recrystallizations gave the same value as that found after two recrystallizations, showing that constancy of isotope concentration had been attained. The product melted at 215–217° and contained 12.46 per cent nitrogen (theory for BTGA, nitrogen 12.34 per cent).

The same procedure was employed for the isolation of BTGA in the control experiment without added chymotrypsin.

Isolation of Benzoyl-L-tyrosinamide (BTA) in Transamidation Experiments—The procedure was similar to that described in the case of BTGA. In separate experiments, the recrystallization of the residual BTA was performed either with 1.7 N NH_4OH , as described previously (1), or with water, but the results obtained were the same. The enzyme experiment reported in Table I led to the isolation of a sample of BTA which melted at 204–205° and contained 9.68 per cent nitrogen (theory for BTA, nitrogen 9.86 per cent).

The authors are greatly indebted to Dr. Henry D. Hoberman and to Mr. Joseph Doolittle for their generous cooperation in the conduct of the isotope analyses.

SUMMARY

Crystalline pancreatic chymotrypsin catalyzes the replacement of the glycine moiety of benzoyl-L-tyrosylglycinamide by isotopic glycine containing N^{15} in the glycine N. This catalysis of a transpeptidation reaction by a crystalline proteinase supports the view that enzymes of this group can catalyze replacement reactions involving peptide bonds.

Cathepsin C, a proteinase found in animal tissues and having a specificity similar to that of pancreatic chymotrypsin, catalyzes the transamidation reaction leading to the replacement of the amide $-\text{NH}_2$ group of glycyl-L-phenylalaninamide by the $-\text{NHOH}$ group of hydroxylamine. A shift in pH from 5.2 to 6.6 markedly increases the extent of transamidation.

Data are presented on the catalysis of transamidation reactions by crystalline pancreatic trypsin and by ficin.

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PHYSICOCHEMICAL STUDIES ON β -AMYLASE*

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In the course of studies in this laboratory on the active groups of enzymes the need arose for substantial quantities of enzymes of a high degree of homogeneity whose activity depends on intact sulfhydryl groups. β -Amylase, recently crystallized by Balls, Walden, and Thompson (1, 2), appeared to be satisfactory, considering the reported yield and the ease of its preparation, but to the authors' knowledge no critical examination of its purity has been reported in the literature.

The purpose of this paper is to ascertain the purity of crystalline β -amylase and to examine some of its physicochemical characteristics.

EXPERIMENTAL

Methods and Materials—The enzyme was assayed by Schwimmer's modification (3) of the method of Kneen and Sandstedt (4), as outlined by Balls *et al.* (2). The unit of β -amylase activity employed is the same as that used by Balls *et al.* (2). Crystalline β -amylase was prepared from a variety of Louisiana Porto Rican red sweet potatoes, by the procedure of Balls *et al.*, except for the following modifications. In the lead acetate precipitation step the use of a Sharples supercentrifuge was found to be more convenient than filtration, as the latter was considerably slower and often yielded turbid filtrates. Secondly, although the activity per mg. of protein N remained constant after three crystallizations, a considerable amount of amorphous material was present even after five or six recrystallizations when the method of Balls *et al.* (2) was used. Separation of the crystalline material from the amorphous substance by differential centrifugation as recommended by these authors did not seem feasible in our hands, as the amorphous material settled on standing along with the crystalline material. Very gradual addition of ammonium sulfate during the recrystallization step (such as over a period of 12 hours) did not effectively prevent the formation of amorphous material. Therefore, during the recrystallization steps, after appropriate washings, the sediment was always redissolved in a minimum amount of water until it was ascertained by microscopic examination that the undissolved residue contained no

* Supported by the Elisabeth Severance Prentiss Fund of Western Reserve University.

more crystals. Saturated ammonium sulfate (pH 3.72 at half saturation at 25°) was added until the solution was made 0.25 saturated with respect to ammonium sulfate (allowing for that occluded in the crystals) and centrifuged at 5000 r.p.m. The clear supernatant was brought to 0.28 saturation with 0.66 saturated ammonium sulfate and immediately centrifuged at high speed. The slight precipitate which formed at this point was discarded and the clear supernatant was then seeded and brought first to 0.33 and then to 0.40 saturation by the addition of the required amount of 0.66 saturated ammonium sulfate over a period of 12 to 18 hours, as outlined by Balls *et al.* (2). When prepared in this manner, after eight crystallizations, the enzyme was entirely free from any amorphous material, as revealed by microscopic examination. Both crystalline forms of the protein were obtained, as previously found by Balls *et al.* (2). The total yield of enzyme in the initial extraction step was only about 30 per cent as much as previously reported (2), and the activity per ml. of extracted juice was about one-half of the reported value. The low yield may have been due to the difference in the type and maturity of the potatoes employed here.

Protein N was determined by micro-Kjeldahl analysis after the protein had been precipitated with an equal volume of 2 N trichloroacetic acid and washed twice with 0.5 N trichloroacetic acid. The dry weight of the protein was determined by drying at 105° for 18 hours after thorough dialysis against cold running, distilled water.

In routine experiments the protein concentration was determined by reading an appropriately diluted aliquot in the Beckman spectrophotometer at 280 $m\mu$ in 1 cm. quartz cells.

A 1 per cent solution, as determined by dry weight, contained 1.35 mg. of protein N per ml.¹ and its absorption in a 1 cm. cuvette at 280 $m\mu$ in the Beckman spectrophotometer was $D = 17.1$. A very slight yellowish color was observed in concentrated solutions of even eight times crystallized preparations. This was also noted by the authors previously mentioned. All the experiments reported in this paper were done with eight times crystallized β -amylase with an average activity of 1153 units per mg. of protein N.

Electrophoretic Examination—Samples of β -amylase were exhaustively dialyzed against the following buffers: veronal buffer, pH 8.59; phosphate buffer, pH 6.36; acetate buffer, pH 4.94; and acetate buffer, pH 3.98. Following dialysis, small samples were removed and assayed for activity.

¹ The N content reported here is somewhat lower than that reported by Balls *et al.* (2). However, in view of the small amount of protein used for dry weight determination and a possible 4 per cent error in the micro-Kjeldahl determinations the value reported here is not necessarily more correct.

It was ascertained that no loss in activity occurred at any of these pH values. Single samples for each buffer were subjected to electrophoretic analysis in the 2 ml. micro cell by means of the Klett model of the Tiselius apparatus. The Longworth scanning method was used to record the moving boundaries photographically. All other conditions of these analyses are presented in the legend of Fig. 1. Electrophoresis was continued until the boundary migrated across the cell. Within the limits of these experiments at the four pH values studied, the protein exhibited a single peak migrating at a uniform rate.

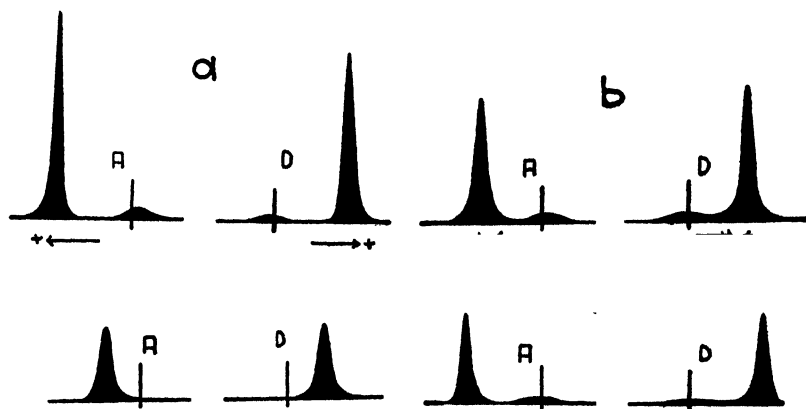


FIG. 1. Electrophoretic patterns of eight times crystallized β -amylase. (a) Veronal buffer, pH 8.59; $\Gamma/2$, 0.1; protein concentration, 13.68 mg. per ml.; time, 9000 seconds; current 0.005 ampere; temperature, 1.38° . (b) Phosphate buffer, pH 6.36; $\Gamma/2$, 0.1; protein concentration, 5.28 mg. per ml.; time, 7200 seconds; current, 0.005 ampere; temperature, 1.35° . (c) Acetate buffer, pH 4.94; $\Gamma/2$, 0.1; protein concentration, 3.05 mg. per ml.; time, 18,000 seconds; current, 0.006 ampere; temperature, 1.38° . (d) Acetate buffer, pH 3.98; $\Gamma/2$, 0.1; protein concentration, 6.79 mg. per ml.; time, 12,780 seconds; current, 0.006 ampere; temperature, 1.35° . Salt boundaries are apparent in (a), (b), and (d).

Isoelectric Point—Interpolation of the curve of electrophoretic mobility as a function of pH (Fig. 2) permitted an approximation of the pH of zero migration, or the isoelectric point. Values of 4.74 and 4.79 were obtained from the curves of the ascending and descending boundaries, respectively.*

Phase-Solubility Curve—For further evaluation of the homogeneity of our sample of β -amylase a phase solubility study was undertaken (5). Three identical experiments were performed by the following procedure. Varying amounts of an exhaustively dialyzed β -amylase stock solution were added to a series of 10 ml. test-tubes containing a mixture of ammonium

sulfate solution and acetate buffer at pH 3.81 so as to give a saturation of 0.45 with respect to ammonium sulfate in 1.66×10^{-2} M acetate buffer, in a final volume of 3 ml. The tubes were shaken for 24 hours in a water bath regulated at $8^\circ \pm 0.01^\circ$. At that time a 1 ml. portion was rapidly

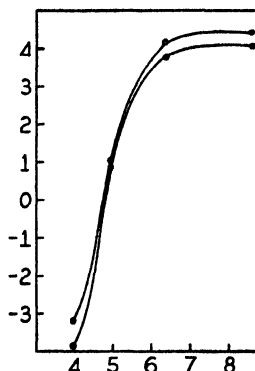


FIG. 2. Variation of electrophoretic mobility with pH. $\circ$, ascending boundary; $\bullet$, descending boundary; abscissa, pH as measured with glass electrode at 25° ; ordinate, mobility in $u \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1} \text{ volt}^{-1}$; temperature, 1.35° .

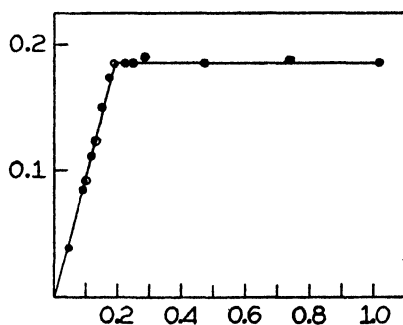


FIG. 3. Solubility curve. Abscissa, protein concentration of the suspension; ordinate, protein concentration of the solution. The concentration is expressed as the light absorption (D) at $280 \text{ m}\mu$ in 1 cm. cells of suitably diluted aliquots (see the text).

removed from each tube and diluted with water to 3 ml. for a later determination of the total protein present in the suspension. The remaining 2 ml. were rapidly centrifuged at 8° and 1 ml. of the clear supernatant was similarly diluted with water to 3 ml. for the determination of the protein concentration in solution. Subsequently, protein concentrations were read in the Beckman spectrophotometer at $280 \text{ m}\mu$ in 1 cm. calibrated cuvettes against a similarly treated blank except for the absence of protein. Fig. 3 represents the results of a typical experiment wherein the light

absorption at 280 $m\mu$ of the total protein present is plotted on the abscissa against that of the protein in solution on the ordinate. The values given are those actually obtained for the dilute solutions after suitable minute corrections for the variations in light paths of the cells.

It is apparent that within the limitations of the experimental technique no indications of the presence of more than one solute absorbing at 280 $m\mu$ was obtained.

Sedimentation Velocity Analysis and Diffusion Constant—A sample of eight times crystallized β -amylase in 0.40 saturated ammonium sulfate was kindly examined by Dr. Sam Sorof of the Department of Physio-

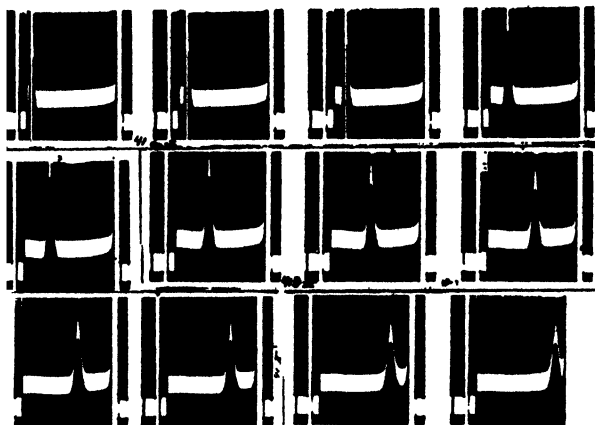


FIG. 4. Velocity sedimentation photographs. The protein concentration was 1.4 per cent, and the rotation speed 59,780 r.p.m.; the temperature of the rotor varied from 20.7–21.9°. Two 4 minute intervals exist between the first three exposures; thereafter photographs were taken every 8 minutes. A 50° bar angle was used throughout. The first exposure was taken 3 minutes after full speed was attained.

logical Chemistry, University of Wisconsin, in a Specialized Instruments Corporation ultracentrifuge. Prior to ultracentrifugation, the sample was dialyzed with stirring against 2000 volumes of 0.15 M NaCl-0.01 M phosphate buffer, pH 6.0, and it was then made up to volume with the above buffer. Further conditions of the ultracentrifuge analysis and photographs of the sedimenting boundaries obtained with the Philpot-Svensson optical system are given in the legend of Fig. 4. Following ultracentrifugation the enzyme was reprecipitated with 2 volumes of saturated ammonium sulfate and returned to this laboratory. An assay performed at that time showed no significant loss in activity per mg. of protein N.

The above determination revealed that about 97 per cent of the protein moved with an S_{20} equal to 8.9 S. In addition a border line impurity,

present to the extent of about 3 per cent, migrated with an average velocity of 3.5 S. In view of the electrophoretic and solubility data there is every reason to believe that the β -amylase constituted the principal component of the two.

The diffusion constant of the protein at a concentration of 0.5 per cent was measured in a 2 cm. electrophoresis cell at 1.40° in the same buffer as employed for ultracentrifugation. The initial boundary was carefully adjusted by an electrolytic compensator and the protein was allowed to diffuse for about 30 hours, photographs being taken at suitable intervals by means of a Philpot-Svensson optical system.

The diffusion constant was calculated from the equation $A^2/(4\pi t(H_m)^2)$ by plotting $1/H_m^2$ against t for each interval of time at which photographs were taken. H_m is the maximum ordinate of each curve and t is the time in seconds (6). The value calculated for the diffusion during the first 24 hours was $D_{20} = 5.77 \times 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1}$.

The molecular weight was calculated from the relation, $\text{mol. wt.} = (RTS_{20})/(D(1 - V_2))$. Assuming a partial specific volume of 0.749 for the average protein at 20° (7), we obtained a value of $152,000 \pm 10$ per cent for the molecular weight.

DISCUSSION

The results presented indicate that eight times crystallized β -amylase shows a high degree of homogeneity.

The phase-solubility studies and electrophoretic analyses revealed the presence of only one protein component. The 3 per cent impurity observed in the ultracentrifuge may not have been apparent in the electrophoretic analyses in view of the lower protein concentrations employed. Alternative possibilities are that the impurity migrates at the same rate as the β -amylase or that it may be a non-protein particle bearing no charge and hence one that would manifest its presence only as a slight boundary disturbance. The reason that this impurity escaped detection in the solubility test might be explained as follows. Since the optical device of the ultracentrifuge measures the refractive indices of the various components, and in the solubility test under our conditions only the ultraviolet absorption was measured, a non-protein impurity or a protein containing little tyrosine or tryptophan would not be detected in the solubility test as employed but would be detected in the ultracentrifuge.

From the molecular weight found the turnover number of the enzyme may be calculated as follows. The purest preparations had an activity of 156,000 units per gm. of protein at pH 4.80 and 30° , in which 1 unit is equivalent to 1 mm of glycosidic linkage hydrolyzed in 10 minutes. Thus 1 molecule of β -amylase with a molecular weight of 152,000 hydrolyzes

$(1.56 \times 10^5 \times 1.52 \times 10^5)/(6.02 \times 10^{23}) \times 6.02 \times 10^{20}$ individual glycosidic bonds in starch in 10 minutes. This corresponds to a turnover number of 2,370,000 per minute at 30°, pH 4.80, one of the highest values ever recorded in the literature.

We are greatly indebted to Dr. Sam Sorof, of the Department of Physiological Chemistry, University of Wisconsin, for the determination of the sedimentation velocity. We also wish to thank Dr. Louis Pillemer of the Institute of Pathology, Western Reserve University, for his valuable discussions, and Mr. Myron O. Schilling, Mrs. C. Coughlin, and Mrs. B. Claus for their generous assistance in the electrophoresis and ultracentrifuge experiments.

SUMMARY

1. Eight times crystallized β -amylase was subjected to electrophoretic analyses, phase rule-solubility studies, diffusion, and ultracentrifugation.

2. The first two of these methods revealed the presence of only one component. An impurity, constituting about 3 per cent of the total material, was detected by ultracentrifugation.

3. β -Amylase was found to have a sedimentation velocity constant of 8.9×10^{-13} second at 20° and a diffusion constant of 5.77×10^{-7} cm.² sec.⁻¹, corresponding to an approximate molecular weight of 152,000 $\pm$ 10 per cent.

4. The isoelectric point determined by electrophoresis is pH 4.74 to 4.79.

5. The number of glycosidic linkages hydrolyzed by 1 molecule of enzyme per minute at 30° and pH 4.80 (turnover number) has been calculated to be 2,370,000.

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AMINO ACID COMPOSITION OF HEMOGLOBINS OF NORMAL NEGROES AND SICKLE-CELL ANEMICS

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The recent electrophoretic experiments of Pauling, Itano, Singer, and Wells (1) have demonstrated that the hemoglobins of normal Negroes and of sickle-cell anemics are unlike; in a buffer of suitable pH the two components of a mixture of these hemoglobins migrate in opposite directions. On the basis of the difference in their isoelectric points, it was concluded that the hemoglobin of sickle-cell anemics has 2 to 4 more net positive charges per molecule than normal hemoglobin. There is evidence that the composition of the heme moiety is the same in both proteins; one hypothesis which has been advanced to explain the dissimilarity of the two hemoglobins assumes a difference in the number or kind of ionizable groups in the molecules, that is, in the acidic or basic amino acids of the globin. Thus, the observed difference in charge might result from a small decrease in the number of acidic amino acids or a small increase in the number of basic amino acids in the molecule of sickle-cell hemoglobin relative to that of normal hemoglobin or from relatively large alterations in amino acid content in which the charges were almost compensated.

The object of the present study was to determine the amino acid composition of the two hemoglobins in order to ascertain the validity of this hypothesis. The analysis was made by means of the methods of starch chromatography which have recently been developed by Moore and Stein (2-6). The hydrolysates of two independently prepared samples of normal carbonmonoxyhemoglobin (to be referred to as N-Hb) and one sample of sickle-cell anemia hemoglobin (SC-Hb) have been analyzed. Approximately ten to fifteen individual determinations of each of seventeen amino acids and ammonia have been made by means of about 70 starch chromatograms.

EXPERIMENTAL

Sources of Hemoglobins—Red blood cells which remained after the removal of the plasma from the whole blood of Negro donors were obtained

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† Contribution No. 1408.

from a commercial laboratory.¹ Individual samples which exhibited no evidence of sickle-cell trait in the diagnostic test of Itano and Pauling (7) were pooled and used in the preparation on N-Hb. Blood from five sickle-cell anemics was used in the preparation of SC-Hb.² All samples were positive in the diagnostic test (7) and, before pooling, were found electrophoretically to be free of normal hemoglobin.

Preparation and Purification of Hemoglobins—With minor modification, the method of Drabkin (8) was used for the crystallization and purification of the proteins. The carbonmonoxyhemoglobin rather than the oxyhemoglobin was isolated and the proteins were kept in this form by saturating each solution with carbon monoxide between successive stages of the purification. Before crystallizations and after the final crystallization, the solutions were centrifuged at $36,500 \times g$ for 30 minutes. After the second crystallization, the crystals were dissolved, and the solution was dialyzed against distilled water with three or four changes per day for 3 days and against doubly distilled water with two changes per day for 2 days. A negative test for phosphate was obtained from the dialyzing water. The final solution was saturated with carbon monoxide, centrifuged at high speed, and stored in brown glass bottles under carbon monoxide at 5°. Samples for the experiments were taken from such stock solutions, which had concentrations of 4 to 10 gm. of Hb per 100 ml. of solution. The two types of Hb showed no evident differences in behavior in this procedure. Stock solutions of two preparations of N-Hb and one preparation of SC-Hb were available.

Criteria of Purity—The electrophoretic homogeneity was used as an initial criterion of purity. The procedure for the electrophoretic experiments was that of Pauling, Itano, Singer, and Wells (1). Both types of Hb showed little evidence of contamination even before crystallization. Representative patterns which were obtained from the stock solutions are shown in Fig. 1. In these experiments the concentrations of Hb were 0.5 to 1.0 gm. per 100 ml. Phosphate buffer of pH 6.95 and 0.1 ionic strength and cacodylate buffer (Longsworth (9)) of pH 6.50 and 0.1 ionic strength were used. The time of electrophoresis was 15 to 20 hours.

The stock solutions were also tested for the presence of ionic iron and phosphate. The protein in a portion of solution was coagulated by heat in the presence of sodium chloride and the coagulated protein was filtered off. A test with thiocyanate showed that less than 0.4 γ of iron (limit of sensitivity) was present in 1 ml. of stock solution; the quantity of protein

¹ We are indebted to the Hyland Laboratories, Los Angeles, for generously supplying us with red blood cells.

² We wish to thank Dr. Harvey Itano of these Laboratories and Dr. Philip Sturgeon of the Children's Hospital, Los Angeles, for having obtained this blood for us.

iron was about 0.3 mg. Tests with ammonium molybdate showed that phosphate was present to the extent of roughly 0.2 mg. per ml.

Concentration of Stock Solutions—The concentration of the Hb solutions was based upon the iron content. Portions of the solutions were evaporated to dryness and the residue was incinerated in a muffle furnace. The quantity of iron was then determined by Wells' adaptation³ of the method of Edmonds and Birnbaum (10). The concentration of the solution in terms of Hb was then calculated on the basis of an iron content in Hb of 0.340

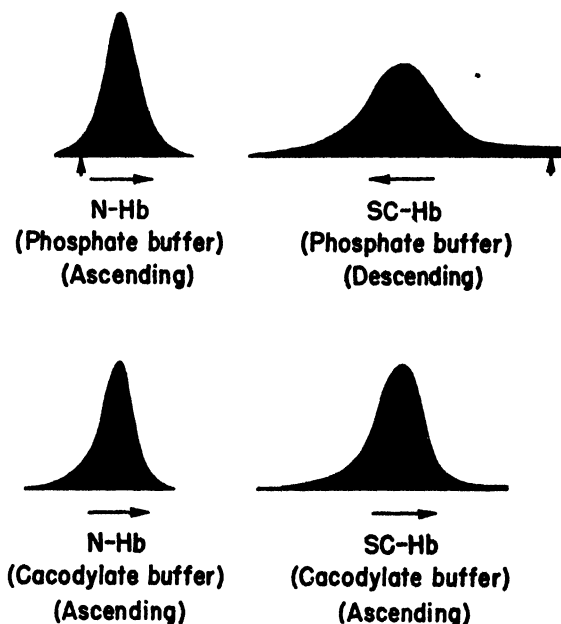


FIG. 1. Longworth scanning diagrams of normal and sickle-cell anemia carbon-monooxyhemoglobins. In the phosphate buffer, the two proteins move in opposite directions. In the cacodylate buffer, the starting boundaries are outside of the range of the drawing.

per cent as determined by Bernhart and Skeggs (11) for N-Hb; this percentage was also assumed to be the iron content of SC-Hb. Confirmation of the concentration as determined in this manner was also obtained from the dry weight of the Hb after evaporation of the solution and from the nitrogen content of the solution. The dry weight of the residue was found by heating at 60° for 24 hours, and then at 100–110° for 12 to 24 hours. The dry weight was about 2.5 per cent greater than would be calculated from the concentration by iron analysis. The nitrogen contents

³ Wells, I. C., to be published.

of the Hb solutions were determined.⁴ From these analyses, and from the iron content of the solutions together with the assumed iron content of Hb (0.340 per cent), the nitrogen content of N-Hb was calculated to be 16.9 per cent and that of SC-Hb to be 16.5 per cent. This nitrogen content of N-Hb is in excellent agreement with the values of 16.9 and 17.0 per cent which are reported by Vickery (12).

Acidic Hydrolyses of Hemoglobins—Acidic hydrolyses were made in relatively dilute solution as suggested by Stein and Moore (3). About 10 ml. of approximately 6 N distilled HCl per 0.1 gm. of protein were used and most of the solutions were refluxed for 16 hours; the first hydrolysate of N-Hb was refluxed for 30 hours. The immediate precipitation which occurs when Hb is mixed with acid makes it advisable to add Hb to the acid with rapid swirling of the solution in order to keep the precipitate as finely divided as possible. During the 1st hour of the hydrolysis, vigorous foaming occurs.

At the end of the period of refluxing, the excess HCl was removed *in vacuo* and water was added and removed by evaporation several times. Early hydrolysates were taken up finally in water; later ones were taken up in 0.1 N HCl in order to hinder oxidation of the methionine. All hydrolysates were stored at 5°.

Basic Hydrolyses of Hemoglobins—About 2 ml. of 5 per cent SnCl_2 in 5 N NaOH per 0.1 gm. of protein were used for the basic hydrolyses, which were carried out under nitrogen in a sealed tube by heating for 16 hours in a boiling water bath. The hydrolysate was then removed to a volumetric flask and neutralized with approximately 6 N HCl. As neutrality is neared, silica and tin compounds precipitate. Excess acid in sufficient quantity to redissolve the tin compounds has no deleterious effect on the chromatograms.

Chromatographic Methods—The starch chromatographic methods of Moore and Stein (2-6) have been so well developed and described that it has been possible to apply them with relatively few modifications. The large number of chromatograms which have been carried out in the present study have permitted an assessment of the usefulness of the various types of developer, an amplification of some of the experiences of Moore and Stein, and some extension of the method.

Two lots of the recommended potato starch obtained from the Amend Drug and Chemical Company have behaved somewhat differently. An appreciable percentage of agglomerates in the second lot will not pass a 60 mesh sieve. The most important difference of this second lot from the first is the fact that certain critical separations, such as glutamic acid from

⁴ Analysis by Dr. Adalbert Elek, Los Angeles. An unpublished modification of the micro-Kjeldahl method was used.

alanine in 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl, are not satisfactory.

Chromatographic columns which were 10 to 11 mm. in diameter and 300 $\pm$ 20 mm. in length were used in all experiments. The effluent volumes at which the zones emerged were in good agreement with those reported by Moore and Stein, if the difference in the sizes of the columns is taken into account. Samples of hydrolysate which were equivalent to 2 to 5 mg. of protein were placed on the column.

Two fraction collectors were used in the present work: one was the commercial model described by Stein and Moore ((3) foot-note 8) and the other had been constructed in these Laboratories. The latter machine moves empty test-tubes under the column after preset periods of time and is not a drop counter like the commercial model. Equally satisfactory results were obtained from chromatograms regardless of which machine was used. Most of the chromatograms were run in duplicate or triplicate at the same time.

n-Butyl Alcohol-Benzyl Alcohol-Water As Developer—When *n*-butyl alcohol-benzyl alcohol-water was used as the developer, the separation of leucine from isoleucine was excellent despite the fact that they are present in the ratio of 75:1.

It has been found that in this developer tryptophan emerges with phenylalanine and is not between phenylalanine and leucine as Stein and Moore ((3) p. 359) have indicated.⁵ Since some tryptophan survives acidic hydrolysis, as will be shown later, uncertainty is thrown upon the content of phenylalanine in the proteins.

As ordinarily carried out, the separation of tyrosine and valine never approached the separation which Stein and Moore (3) achieved. Part of the difficulty may have been caused by the greater ratio of valine to tyrosine, about 3:1, in the Hb samples as compared to about 1.5:1 in their experiments. However, a more important factor is the temperature at which the chromatograms are run. Although no records were kept, room temperature probably has ranged from 20–30° in the course of the work. Recently, at the suggestion of Dr. Stein, water-jacketed chromatographic tubes have been used and the column has been maintained at about 15°. Under these conditions the separation of tyrosine and valine is complete and excellent. At this temperature the water content of the solvent must be reduced from 144 ml. as suggested ((3) p. 353) to 130 ml. in order to preserve undersaturation.

⁵ Dr. William H. Stein has suggested (private communication) that the disagreement may be caused by small differences in the water content of the developer, since it is known that the water content greatly influences the behavior of the aromatic amino acids.

n-Butyl Alcohol-17 Per Cent 0.5 *N* HCl As Developer—Because initial attempts to separate tyrosine from valine with the preceding developer were unsuccessful, most of the results which will be reported were obtained in the following manner. Development with *n*-butyl alcohol-17 per cent 0.5 *N* HCl was used to separate tyrosine from the mixture of valine and methionine (3). Methionine, however, forms a well separated zone when *n*-butyl alcohol-benzyl alcohol-water is the developer. Thus, valine may be obtained by difference. Satisfactory separations of tyrosine from valine and methionine were obtained on the first lot of starch but not on the second. This developer also furnishes values for phenylalanine and the sum of leucine plus isoleucine. Tryptophan emerges at effluent volumes between the peaks of phenylalanine and leucine plus isoleucine.

1:2:1 *n*-Butyl Alcohol-*n*-Propyl Alcohol-0.1 *N* HCl and 2:1 *n*-Propyl Alcohol-0.5 *N* HCl As Developers—These developers generally are satisfactory although hemin and its degradation products cause some interference after the change from the first to the second solvent. At the beginning of the chromatogram some brown material passes immediately through the column. After the solvent is changed, one brown zone washes down with the front of increased water content and produces a marked artifact zone ((5) p. 60) and a second with the front of increased acid. Although this second zone frequently is contained in a fraction of the serine, it does not interfere with its determination. This fact was proved by chromatograms in which the serine was washed through without changing the solvent; the results of both types agreed.

2:1:1 *tert*-Butyl Alcohol-*sec*-Butyl Alcohol-0.1 *N* HCl As Developer—Adequate although not complete separations of glutamic acid from alanine were obtained on the first lot of starch; indeed, there was even a considerable separation of proline from the glutamic acid. More recently, the use of 3:1 *tert*-butyl alcohol-0.1 *N* HCl as suggested (5) has been necessary although the separation is not complete. In either solvent, proline is present in the zone of glutamic acid and its contribution to the optical density in the colorimetric procedure must be taken into account in the calculation ((5) p. 64). Values for phenylalanine and the sum of leucine plus isoleucine can be obtained from these chromatograms.

0.1 *N* HCl As Developer—This solvent may be used for the separation of tryptophan as a well formed zone which is well separated from the other amino acids (5), but Moore and Stein have not applied the method to the determination of tryptophan in a basic hydrolysate. Experiments showed that the presence of NaCl and SnCl₂, which would be present if a neutralized basic hydrolysate were chromatographed directly, did not alter the chromatographic properties of tryptophan and the other amino acids in 0.1 *N* HCl as developer. Under the conditions of basic hydrolysis which

have been given above, the recovery of tryptophan alone was 98 per cent and that of tryptophan when added to the solution of protein before hydrolysis was 104 per cent. Satisfactory values for tyrosine may be obtained with this developer. Tyrosine forms a distinct peak on the trailing edge of the zone which contains most of the amino acids; it emerges just before tryptophan (5).

Recovery Experiments—In order to determine whether or not all procedures were being carried out with no more than the expected error, recovery experiments were made with about ten amino acids. The recovery was determined both from synthetic mixtures and after addition of known quantities to a hydrolysate before it was placed on the column. With one major exception the results generally fell within the range of 100 ± 5 per cent which Moore and Stein give as reasonable limits for the method. Aspartic acid, however, has given somewhat unsatisfactory results. When it was chromatographed alone, recoveries of 106, 102, 106, and 100 per cent were obtained after correction for 6 per cent esterification ((5) p. 71); it seems as if esterification occurred to the extent of only 3 per cent in these experiments. The recovery of aspartic acid added to a hydrolysate was 113 and 118 per cent if 6 per cent esterification is assumed. No reasonable explanation has been found for this abnormal behavior, nor can it be said with certainty that the values which will be reported for aspartic acid are high to the extent of approximately 15 per cent. In view of the uncertainties, the results of aspartic acid have been calculated by assuming 6 per cent esterification.

Photometric Ninhydrin Methods—The colorimetric methods as described by Moore and Stein (4) were followed with relatively minor modification. A large set of matched photometer tubes has not been used; instead a single photometer tube was used for all readings of optical density. After the reaction had been completed and the mixture diluted in the test-tube in which the fraction was collected, the solution was poured into the photometer tube, the optical density was read, the solution was poured out, the photometer tube was drained, and the operation was carried out similarly with the next fraction. Silicone-coated tubes to prevent creeping have not been used, because the extent of the creeping was too slight to interfere with thorough mixing of the sample. Because of the greater availability of isopropyl alcohol, the dilutions have been made with 1:1 isopropyl alcohol-water rather than with 1:1 *n*-propyl alcohol-water. Tests showed that this substitution was without effect. Interference from ammonia in the air has not constituted a problem in this work.

The color yields which are reported by Moore and Stein (4, 5) have been used without modification, because tests with about six amino acids gave results within 1 per cent of theirs.

TABLE I

Individual Values from Chromatographic Analyses of Hydrolysates of Normal Human Carbonmonoxyhemoglobin

Results in gm. of amino acid per 100 gm. of COHb.

Chromatogram No..	First hydrolysate						Second hydrolysate			
	Developer, <i>n</i> -butyl alcohol-benzyl alcohol-water									
	581	582	583	584	593	594	619	620	621	622
Phenylalanine	7.61	7.81	7.59	7.54	7.95	7.63	7.78	7.55	7.73	7.88
Leucine	17.05*	15.67	14.90	15.46	14.67	15.45	14.92	14.54	15.04	14.91
Isoleucine	0.27	0.24	0.20	0.15	0.15	†	0.26	0.18	0.29	0.15
Methionine	1.17	1.38	0.90*	1.24	1.44	1.37	1.60	1.34	1.59	1.29

Chromatogram No..	Developer, <i>n</i> -butyl alcohol-17 per cent 0.5 N HCl									
	585	586	587	588	589	590	623	624		
Leucine + isoleucine	15.05	14.71	14.71		15.19	15.07	15.18	14.33		
Phenylalanine	7.63	7.39	7.71		7.75	7.90	7.68	7.51		
Valine	10.93	10.56	10.60		10.58	10.58	11.14*	10.37*		
Tyrosine	4.56*	3.41	3.09	2.90	3.00	2.78	2.65*	2.88*		

Chromatogram No. .	Developer, <i>tert</i> -butyl alcohol- <i>sec</i> -butyl alcohol-0.1 N HCl									
	561	562	563	575	578		625	626		
Leucine + isoleucine	14.66	15.12	15.37	14.93	14.29		15.18	14.90		
Phenylalanine	9.17*	8.00	7.27	7.46	7.17		7.85	7.85		
Glutamic acid	7.22	7.37			7.09		7.54*	6.99		
Alanine	10.29	10.32	9.51	10.00	10.77		10.51*	10.23		

Chromatogram No. .	Developer, <i>n</i> -butyl alcohol- <i>n</i> -propyl alcohol-0.1 N HCl and <i>n</i> -propyl alcohol-0.5 N HCl									
	559	560	564	565	566	571	577	627	628	
Proline	4.26	4.38	4.26	4.36	4.41	4.49	4.22	4.55	4.43	
Threonine	6.09	5.58		5.80	5.63	5.85	5.99	6.10	5.77	
Aspartic acid	11.04	11.00			10.95		10.24	11.33	11.54	
Serine	5.07		5.07	5.02	5.34*	5.07	5.07		5.02	
Glycine	5.02	4.81	4.73	4.68	5.00	4.83	5.12	4.81	4.64	
Ammonia	0.95	0.92	0.88	0.93	0.95	0.98	0.97	0.86	0.82	
Arginine	3.42	3.64	3.07	3.17	3.20	2.78		3.43	3.56	
Lysine	9.49	10.12	9.37	9.41	9.83	9.76		9.51	9.61	
Histidine	8.74	8.64	8.24	8.00	8.83	8.83		8.15	8.07	

Chromatogram No..	Developer, 0.1 N HCl									
	642	643	644				647	648		
Tyrosine	3.04	2.68	2.97				2.95	3.08		
Tryptophan	1.45	1.44	1.57				1.50	1.51		

TABLE I—*Concluded*

* Stricken from further consideration mainly because of chromatographic difficulties but sometimes because of wide deviation from other results; not included in the averages in Tables III and IV.

† Individual values which are lacking from the results of a given chromatogram could not be calculated for various reasons; for example, accidental loss of fractions.

In the determination of tryptophan with 0.1 N HCl, the blank should be calculated from the blanks before the first main zone and those after the emergence of tryptophan. The blanks between the tyrosine and tryptophan usually are slightly above this level.

Results

Seventeen amino acids and ammonia have been shown by the chromatographic analyses to be present in the two types of Hb. These compounds were identified by the relative positions of the peaks. There is no evidence of the presence of other than the commonly occurring amino acids. Hydroxyproline is not detectable by these methods and no attempt was made to find it by other means. The determination of cystine is one of the more difficult determinations of starch chromatography; this amino acid emerges after the histidine over a long series of fractions and hence its concentration in each fraction is rather low. Some chromatograms showed evidence of a zone in the position to be expected for cystine, but the optical densities were low in the colorimetric determination and attempts to calculate the quantity of cystine were unwarranted. Hence, cystine probably is present but the relative amount is very small.

Tables I and II list the individual results of the analyses for the various amino acids by means of the several developers.

Table III presents various types of information which are deducible from the data of Tables I and II and are valuable in judging the similarity or dissimilarity in the composition of the two proteins. The recorded average gm. of amino acid per 100 gm. of protein is the average of all acceptable values of a given amino acid in both hydrolysates of each type of Hb. Table III also includes the standard deviation and spread of the results as well as a calculation of the number of residues of each amino acid per molecule of 66,700 molecular weight and of the number of residues equivalent to one standard deviation.

In Table IV, the average values are used for the calculation of gm. of amino acid residue per 100 gm. of protein and for the gm. of nitrogen per 100 gm. of protein. In Table IV, the quantity of leucine is that determined in the developer in which it is completely separated from isoleucine; the quantities of phenylalanine, tyrosine, and valine are the averages of all acceptable values from more than one developer. The results are also

TABLE II

Individual Values from Chromatographic Analyses of Hydrolysates of Sickie-Cell Anemia Carbonmonoxyhemoglobin

Results in gm. of amino acid per 100 gm. of COHb.

First hydrolysate							Second hydrolysate					
Developer, <i>n</i> -butyl alcohol-benzyl alcohol-water												
Chromatogram No...	659	660	661				707*	708*	709*	713	714	715
Phenylalanine	7.40	7.68	7.50				7.73	7.58	7.36	7.65	7.74	7.59
Leucine	14.34	14.37	14.25				14.79		14.45	14.74	14.49	14.52
Isoleucine	0.16	0.16	0.22				0.27	0.40†	0.09†	0.15	0.14	0.23
Methionine	1.07	1.37	1.43				1.75	1.71	1.46	1.37	1.19	1.54
Tyrosine							3.04	3.07	2.94			
Valine							10.86	10.28	10.21			

Developer, <i>n</i> -butyl alcohol-17 per cent 0.5 N HCl												
Chromatogram No.	668	669	670									
Leucine + isoleucine	14.29	13.76	14.23									
Phenylalanine	7.41	7.32	7.19									
Valine	10.69†	10.13†	9.71†									
Tyrosine	2.98†	2.48†	2.61†									

Developer, <i>tert</i> -butyl alcohol- <i>sec</i> -butyl alcohol-0.1 N HCl or <i>tert</i> -butyl alcohol-0.1 N HCl‡												
Chromatogram No..	665	666	667	686	701	703						
Leucine + isoleucine	13.85	14.53	14.37	14.20								
Phenylalanine	7.64	7.73	7.79	7.53								
Glutamic acid	8.07†	6.83†	6.96†	7.04	7.14	7.48						
Alanine	9.09†	10.17†	9.68†	10.09	10.22	10.18						

Developer, <i>n</i> -butyl alcohol- <i>n</i> -propyl alcohol-0.1 N HCl and <i>n</i> -propyl alcohol-0.5 N HCl§												
Chromatogram No..	662	663	664	690	691	692	704	705	706			
Proline	4.20	4.23	4.20	4.27	4.42	4.42	4.41	4.29	4.41			
Threonine	6.03	5.69	6.00	6.01	6.07	6.11	5.99	5.99	6.11			

TABLE II—*Concluded*

Chromatogram No...	First hydrolysate					Second hydrolysate							
	662	663	664	690	691	692	704	705	706				
Developer, <i>n</i> -butyl alcohol- <i>n</i> -propyl alcohol-0.1 N HCl and <i>n</i> -propyl alcohol-0.5 N HCl§— <i>Concluded</i>													
Aspartic acid	11.34	11.13	11.10	11.65	11.87	11.59	11.30	11.39	11.70				
Serine	5.47	5.53		5.64	5.48	5.42	5.28	5.37	5.28				
Glycine	4.79	4.76	4.70	5.11		4.98							
Ammonia	0.86	0.86	0.82										
Arginine	3.74	3.28	3.28										
Lysine	9.77	9.58	9.64										
Histidine	8.59	8.28	8.56										
Developer, 0.1 N HCl													
Chromatogram No...	672	673	674										
Tyrosine*	3.13	2.77	3.11										
Tryptophan	1.48	1.62	1.69										

* These chromatograms only were run at a lower temperature in order to make possible the determination of tyrosine and valine.

† See the asterisk foot-note of Table I.

‡ Chromatograms 686, 701, and 703 were run with the second solvent.

§ Chromatograms 690 to 692 and 704 to 706 were run with the first solvent only, since it was desired to obtain values for serine without interference from brown contaminants.

compared with the quantities which have been reported in the literature for the amino acid content of human globin and Hb.

Although the quantities have been reported to two to four digits, a consideration of the standard deviation and spread of the results shows clearly that in few instances does the last figure have real significance. Because the main purpose of this study is a comparison of the amino acid content of the two types of Hb, up to four significant figures have been retained because rounding off the values in many instances increases or decreases the apparent similarity or dissimilarity of the contents to an unwarranted extent. In like manner, the calculation of the number of residues of amino acid per molecule of hemoglobin must be treated with reserve. The results of such a calculation may give added insight into the general composition of the molecule, but if due regard is given to the present state of the development of analytical methods, and to the possible destruction of amino acids during hydrolysis, it is realized that the number of residues

TABLE III

Average Quantity of Amino Acids, Standard Deviation of Results, and Other Values Deducible from Tables I and II

In general, the last figure given is not significant (see the text).

Constituent	Average gm amino acid per 100 gm. COHb		Standard deviation		Spread of results, per cent of average		No. of residues per 66,700 mol. wt., calculated from average		Residues per unit standard deviation	
	Normal	Sickle-cell	Normal	Sickle-cell	Normal	Sickle-cell	Normal	Sickle-cell	Normal	Sickle-cell
Alanine	10.19	10.16	0.41	0.07	12	1	76.4	76.1	3.07	0.52
Ammonia	0.92	0.85	0.05	0.02	17	5	36.1	33.4	1.96	0.78
Arginine	3.28	3.43	0.28	0.27	26	13	12.6	13.1	1.07	1.04
Aspartic acid	11.02	11.45	0.44	0.27	12	7	55.3	57.4	2.21	1.35
Cystine										
Glutamic acid	7.17	7.22	0.16	0.23	5	6	32.5	32.8	0.73	1.04
Glycine	4.85	4.87	0.16	0.17	10	8	43.1	43.3	1.42	1.51
Histidine	8.44	8.48	0.36	0.17	10	4	36.3	36.5	1.55	0.73
Isoleucine, Developer 1*	0.21	0.19	0.06	0.05	67	68	1.1	1.0	0.31	0.25
Leucine, Developer 1	15.06	14.49	0.38	0.19	8	4	76.7	73.8	1.93	0.97
" + isoleucine										
Developer 2*	14.92	14.24	0.36	0.29	7	5	76.0	72.5	1.83	1.48
" 3*	14.89	14.09	0.32	0.29	6	4	75.8	71.7	1.63	1.48
Lysine	9.64	9.66	0.25	0.10	8	2	44.0	44.1	1.14	0.46
Methionine	1.38	1.43	0.15	0.22	31	48	6.2	6.4	0.67	0.98
Phenylalanine†										
Developer 1	7.71	7.58	0.15	0.14	5	5	31.2	30.6	0.61	0.57
" 2	7.60	7.67	0.35	0.11	11	3	30.7	31.0	1.41	0.44
" 3	7.65	7.31	0.18	0.11	7	3	30.9	29.6	0.73	0.44
Proline	4.39	4.32	0.11	0.10	8	5	25.5	25.1	0.64	0.58
Serine	5.05	5.43	0.03	0.12	1	7	32.1	34.5	0.20	0.76
Threonine	5.85	6.00	0.20	0.13	9	7	32.8	33.6	1.12	0.73
Tryptophan	1.49	1.60	0.05	0.11	9	13	4.9	5.2	0.16	0.36
Tyrosine										
Developer 1		3.02		0.07		4		11.1		0.26
" 3	3.04		0.25		21		11.2		0.92	
" 4*	2.94	3.00	0.16	0.20	14	12	10.8	11.1	0.59	0.74
Valine										
Developer 1		10.45		0.36		6		59.6		2.05
" 3	10.65	(10.18)‡	0.13	(0.49)	3	(19)	60.7	(58.0)	0.74	(2.79)

* Developer 1, *n*-butyl alcohol-benzyl alcohol-water; Developer 2, *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl; Developer 3, *n*-butyl alcohol-17 per cent 0.5 N HCl; Developer 4, 0.1 N HCl.

† If contamination with tryptophan is taken into account, the values for phenylalanine would be decreased by 0.3 to 0.4 gm. per 100 gm.

‡ Of doubtful reliability, but included for comparison.

TABLE IV

Composition of Normal and Sick-Cell Anemia Carbonmonoxyhemoglobins
 In general, the last figure given is not significant (see the text).

Constituent	Normal carbon-monooxyhemoglobin			Sickle-cell carbon-monooxyhemoglobin			Values from literature, gm. amino acid per 100 gm. protein
	Gm. amino acid per 100 gm. COHb	Gm. residue per 100 gm. COHb	Gm. nitrogen per 100 gm. COHb	Gm. amino acid per 100 gm. COHb	Gm. residue per 100 gm. COHb	Gm. nitrogen per 100 gm. COHb	
Alanine	10.19	8.13	1.60	10.16	8.11	1.60	9.9 (13), 9.95 (14)
Ammonia	0.92		0.76	0.85		0.70	.
Arginine	3.28	2.94	1.06	3.43	3.08	1.10	3.44* (15), 3.44* (16), 4.21 (12)
Aspartic acid.	11.02	9.53	1.16	11.45	9.90	1.21	
Cystine†							0.81* (15), 0.84* (16), 1.05-1.35 (17), 1.13* (18), 1.21* (19), 1.21* (20)
Glutamic acid .	7.17	6.30	0.68	7.22	6.34	0.69	
Glycine	4.85	3.69	0.91	4.87	3.70	0.91	4.47 (14)
Histidine	8.44	7.46	2.29	8.48	7.50	2.30	7.5* (15), 8.09 (12), 8.55* (16)
Isoleucine . . .	0.21	0.18	0.02	0.19	0.16	0.02	0 (21), 0.29* (22), <0.2-<0.4 (14), 0.7 (23)
Leucine	15.06	13.00	1.61	14.49	12.50	1.55	14.7 (24), 16.0 (25), 17* (22) 17.1 (13)
Lysine	9.64	8.45	1.85	9.66	8.47	1.85	9.18* (16)
Methionine . . .	1.38	1.21	0.13	1.43	1.26	0.13	1.23* (19), 1.32 (21), 1.35-1.69 (17), 1.52* (20)
Phenylalanine ‡	7.66	6.83	0.65	7.55	6.73	0.64	7.90* (15), 8.40 (26)
Proline	4.39	3.70	0.53	4.32	3.64	0.53	4.94 (14)
Serine	5.05	4.19	0.67	5.43	4.50	0.72	
Threonine	5.85	4.97	0.69	6.00	5.09	0.70	5.05-6.25 (27), 6.9* (15)
Tryptophan . . .	1.49	1.36	0.20	1.60	1.46	0.22	1.32,* 1.42* (15), 2.30* (16)
Tyrosine	2.99	2.69	0.23	3.01	2.71	0.23	2.82* (16), 3.04,* 3.44* (15)
Valine	10.65	9.01	1.27	10.45	8.84	1.25	9.12 (25), 10.1 (13), 12.2 (22)
Total amino acids	110.24	93.64	16.31	110.59	93.99	16.35	
Heme, calculated		3.8	0.35		3.8	0.35	
Final total		97.4	16.66		97.8	16.70	
N by analysis			16.9			16.5	
% accounted for		97.4	98.6		97.8	101.2	

* Human globin was analyzed; for other literature values, hemoglobin was used.

† Cystine (or cysteine) is probably present but the quantitative determination was unsatisfactory.

‡ Not corrected for contamination with tryptophan.

of a given amino acid thus arrived at is subject to uncertainties which are sometimes of considerable magnitude.

Comparison of Amino Acid Content of Hemoglobins

The information in Tables I, II, and III has been studied on the basis of the following criteria in order to establish in what particulars the compositions of the proteins are or are not identical. (1) Obvious agreement or disagreement with respect to the average quantity of each individual amino acid. Each amino acid may be assigned to one of three categories: its quantities in the two proteins are apparently either the same, different, or questionably the same or different. (2) The interspersions of individual values from both proteins, that is, inspection to determine whether or not all values seem to be distributed normally and to belong to the same population. (3) Inspection of the ratio: difference in the averages over standard deviation. (4) Comparison of the difference in the averages for each protein with the differences observed in duplicate hydrolysates of the same protein. (5) Comparison of the difference in the averages expressed in terms of the number of amino acid residues per molecule in the protein.

With respect to the first criterion, the seventeen amino acids and ammonia may be divided into three categories as follows: (a) Quantities apparently the same in the two hemoglobins, *i.e.* alanine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, proline, and tyrosine. (b) Quantities apparently different in the two hemoglobins, *i.e.* aspartic acid, leucine, serine, and valine. (c) Quantities questionably the same or different, *i.e.* ammonia, arginine, phenylalanine, threonine, and tryptophan.

All other criteria serve to substantiate the conclusion that the quantities of alanine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, proline, and tyrosine are the same in both proteins.

Let us now consider individually those amino acids whose quantities in the two proteins are either apparently different or questionably so.

Aspartic Acid—Five of the nine determinations of aspartic acid in SC-Hb fall within the range of values from N-Hb and the remainder lie above the upper limit. Aspartic acid has shown a tendency to give a rather wide spread of results and a large standard deviation. Indeed, the agreement of determinations from the two hydrolysates of N-Hb is not very satisfactory. The average for N-Hb can be raised from 11.02 to 11.18 gm. per 100 gm. by rejecting the one very low value, and this change brings the average into considerably better agreement with that of 11.45 from SC-Hb. Consideration must also be given to the fact that the average from SC-Hb (11.45) agrees well with that from the second hydrolysate of N-Hb (11.44). In addition to these statistical factors, it should be noted that the determination of aspartic acid is subject to error because of esteri-

fication which requires a correction factor in the calculations and, as already described, there was difficulty in the recovery of aspartic acid added to hydrolysates; these subjects have been discussed in the experimental part. In view of these uncertainties, it seems probable that the difference in the content of aspartic acid is not significant.

Leucine—For some unexplained reason, the value found for the sum of leucine plus isoleucine from a given protein is always less than that found for leucine alone although leucine constitutes more than 98 per cent of the mixture. The individual values for leucine or the sum of leucine plus isoleucine in SC-Hb fall below or in the lower part of the range of values from N-Hb. The standard deviation for the determination of leucine seems to be about 0.3 gm. per 100 gm. of protein; the averages differ by about 2 standard deviations, a consequence which is of low probability if the leucine contents of the two proteins actually are identical. This apparently significant difference was observed when the results from the first hydrolysate of SC-Hb were compared with those from N-Hb. In order to confirm or disprove the significance of this difference a second hydrolysis of SC-Hb was made and analyzed. Again, the average content of leucine was found to be lower than in N-Hb, although not quite as low as in the first hydrolysate of SC-Hb. On the basis of these results, we may conclude that the hydrolysates differ in content of leucine and that the hydrolysates of SC-Hb contain less leucine than those of N-Hb. The bearing of this conclusion upon the compositions of the two types of Hb is discussed in a subsequent section.

Serine—From reasoning of the same sort as that used in the case of leucine, we conclude that the hydrolysates of SC-Hb contain significantly more serine than the hydrolysates of N-Hb.

Valine—Individual determinations of valine in the first hydrolysate of SC-Hb agreed poorly, probably mainly because the separations of valine plus methionine from tyrosine were not very satisfactory. In spite of this, valine appeared to be present in significantly lesser amount in hydrolysates of SC-Hb than in those of N-Hb. The determinations by a different method in the second hydrolysate were chromatographically excellent and gave individual values of 10.86, 10.28, and 10.21 gm. per 100 gm. of protein, as compared with an average of 10.18 from the first hydrolysate and of 10.65 from N-Hb. Although the individual results for SC-Hb still show a wide spread, their evaluation as in the case of leucine indicates some probability that the hydrolysates of SC-Hb contain significantly less valine than do those of N-Hb. Because of the wide spread of the results, this statement cannot be made with the same degree of confidence as in the case of leucine.

Ammonia—The distribution of individual values is such that those of

SC-Hb and the second hydrolysate of N-Hb agree well but are below the range of results from the first hydrolysate of the N-Hb. Since the ammonia which is determined by the analysis arises not only from amide groups but also from decomposition of the protein during hydrolysis, the observed difference may have resulted because the first hydrolysate of the N-Hb was refluxed for 30 hours whereas the others were refluxed for 16 hours. The difference in the time of hydrolysis had very little effect in the analyses for other constituents; inspection will show that some averages for the amino acids of the second hydrolysate of N-Hb are higher and some are lower as compared to the first hydrolysate. However, decomposition of the protein to the extent of 1 per cent would result in the observed difference in the ammonia of the two hydrolysates, but definite detection of so little destruction is beyond the limits of the method. Certainly the analyses for ammonia have not demonstrated that the amide content of the two proteins is different.

Arginine—The difference in the averages for arginine is caused by one value from SC-Hb which departs far from the average, whereas the others are exactly the same as the average from N-Hb. The standard deviation and spread of results are large in this determination, and thus the observed difference in the averages is not significant.

Phenylalanine—The determination of phenylalanine is rendered uncertain because of contamination of the zone with tryptophan which survives the acidic hydrolysis. Analyses for tryptophan were made in the second hydrolysates of both types of Hb; the content in N-Hb was 0.56 gm. per 100 gm. of protein and in SC-Hb was 0.79 and 0.88. From this result it might be expected that the content of phenylalanine uncorrected for tryptophan would be higher in SC-Hb than in N-Hb. On the contrary, most of the analyses indicated the reverse. Of the three types of chromatograms by means of which phenylalanine may be determined, one set of results indicates no difference in content, one a very probable difference, and the third gives inconclusive results. Unequivocal conclusions are difficult but it seems probable that no difference in the phenylalanine content of the two proteins has been demonstrated.

Threonine—The results for threonine from N-Hb are distributed rather evenly over an appreciable range and are not closely grouped; six from SC-Hb are grouped somewhat above the average for N-Hb, while the others deviate both above and below this group. Six of the nine results from the SC-Hb fall within 1 standard deviation of the average and the difference in the averages is slightly less than 1 standard deviation. Since a relatively large number (seventeen) of determinations have been made and the differences are on the borderline of what is usually considered statistically significant, it may be that the quantity of threonine in hydrolysates of SC-Hb is slightly greater than in hydrolysates of N-Hb.

Tryptophan—The agreement of results from two hydrolysates of N-Hb is excellent, and hence the difference shown by SC-Hb might on cursory inspection seem to be significant. However, because the content of tryptophan in the proteins is small, this relatively large apparent percentage difference in the two is negligible in terms of residues of amino acid. It may therefore be concluded that the contents of tryptophan are the same.

Conclusions—It has been suggested (1) that the observed electrophoretic differences of SC-Hb and N-Hb might be ascribed to the presence of fewer acidic amino acids or more basic amino acids in SC-Hb. The present study gives no evidence of differences in the content of arginine, histidine, lysine, and glutamic acid. Although there is an apparent difference in the content of aspartic acid and ammonia (or amides), it has been concluded that this difference is not real. However, should this conclusion be false, there would be more aspartic acid and less ammonia (fewer amides) in SC-Hb, the opposite of that necessitated by the hypothesis.

The analytical results have established with reasonable certainty that hydrolysates of SC-Hb contain less leucine and more serine and perhaps less valine and more threonine than do hydrolysates of N-Hb. The extent of these differences is a matter of much greater uncertainty. Presumably, the difference is caused by actual dissimilarity in the amino acid content of the intact proteins, although it might arise if the same number of residues of amino acids were arranged differently in the polypeptide chains of the two proteins and as a result the destruction during hydrolysis was of a different degree in the individual proteins. The commonly adopted correction of Rees (28) for the destruction of serine and threonine during hydrolysis has not been applied to the results of this study.

The question may be asked, can the dissimilarity in the electrophoretic properties of N-Hb and SC-Hb be explained in terms of the apparent smaller content of leucine and valine and greater content of serine and threonine in SC-Hb? A lesser content of leucine and valine and a greater content of serine and threonine would bring about a decrease in the non-polar side chains and an increase in the polar side chains of the SC-Hb. Since the hydroxyl groups of serine and threonine are able to form good hydrogen bonds, the possibility exists that the presence of added numbers of these amino acids would cause the polypeptide chains of SC-Hb to fold or coil in a different manner than they do in N-Hb. In this way in SC-Hb as compared to N-Hb, charged groups which are responsible for the electrophoretic differences might be brought into play or removed from action. Such an explanation is also applicable if the proteins differ only in the arrangement of identical numbers of amino acid residues along the polypeptide chain. The folding or coiling of polypeptide chains would also be greatly influenced by the presence of amino acids which possessed a D configura-

tion; since starch chromatography does not distinguish between configurations, the merits of this possibility cannot be assessed.

In conclusion, then, it may be said that the cause of the observed electrophoretic differences in N-Hb and SC-Hb does not reside in their content of the basic and acidic amino acids; it seems likely that they are to be ascribed to differences in the folding or coiling of the molecule.

Total Composition of Proteins

Table IV summarizes the information of the preceding tables and makes a comparison with information already published.

In these types of Hb, approximately 4 per cent of the weight of the protein is in the form of the heme. The remaining 96 per cent has been largely accounted for by the amino acids as determined. There is surprising concordance in the extent to which the weight of the protein is accounted for in the two hemoglobins. The nitrogen recovery of 98.6 per cent from N-Hb compares well with the weight recovery of 97.4 per cent. Because the weight recoveries of the two hemoglobins agree so well, the nitrogen recovery of 101.2 per cent from SC-Hb casts suspicion on the reliability of the nitrogen analysis; the percentage of nitrogen in SC-Hb may well be about 16.9. As previously noted, the contents of serine and threonine have not been corrected for destruction during hydrolysis (28). In view of the errors involved, the recoveries are entirely satisfactory.

There can be little doubt that the two hemoglobins have essentially the same minimum molecular weight, since the quantity of isoleucine in each is such that there can be only 1 residue of this amino acid in a molecular weight of about 67,000.

Basic hydrolysates contain 2 or 3 times as much tryptophan as do acidic hydrolysates. Under the conditions of basic hydrolysis which were used, tryptophan alone or added to the protein before hydrolysis is not destroyed. This test of a satisfactory hydrolytic procedure is open to question on the basis of the work of Spies and Chambers (29), which indicates that peptide-linked tryptophan is partly destroyed during alkaline hydrolysis by peptide-linked cystine, serine, and threonine. On the other hand, equivalent amounts of tyrosine were found in both acidic and basic hydrolysates in the present study.

Numerous investigators (12-27) have published fragmentary analyses of the amino acid content of human Hb. There is often reasonable agreement of the results although they were obtained from many samples of globin or Hb. Birkofer and Taurins (17) report differences in the methionine and cystine content of the Hb of different individuals. The results of the present study usually agree with those of at least one other determination. One of the most recent partial analyses of Hb is that of Keston, Udenfriend, and Cannan (14), in which alanine, glycine, isoleucine, and

proline were determined by the carrier technique. There is excellent agreement between their value for alanine and the present one, and the quantity of isoleucine is of the same magnitude, but the values for glycine and proline do not agree so well. The present determination of isoleucine substantiates previous reports of the presence of this amino acid in a very small amount; the usual absolute amount in the samples chromatographed was of the order of 10 to 15 γ , but even this small quantity produced a definite peak in chromatograms of the hydrolysate and of known mixtures and was further proved to be present in a special chromatogram in which the load on the column was tripled. Cystine (or cysteine) is probably present in Hb, as the work of numerous investigators has shown, but the amount is so small that its quantitative determination by starch chromatography was unsatisfactory. The content of ammonia, aspartic acid, glutamic acid, and serine is reported for the first time.

Porter and Sanger (30), by the use of 2,4-dinitrofluorobenzene, have found forty-three free ϵ -amino groups of lysine per molecule of adult human Hb. The result is in unusual agreement with the 44 residues of lysine which are indicated by the present analysis.

SUMMARY

Hemoglobins from the blood of normal Negroes and of sickle-cell anemics have been quantitatively analyzed for seventeen amino acids and ammonia by the methods of starch chromatography. The results indicate that the two hemoglobins do not differ with respect to their content of basic and acidic amino acids but that hemoglobin from sickle-cell anemics probably contains slightly less leucine and more serine and possibly less valine and more threonine. Although the amino acids in which the proteins seemingly differ cannot in themselves contribute directly to the net charge on the proteins and thereby influence the electrophoretic properties, they may affect the folding or coiling of the polypeptide chains and in this way either bring into play or remove from action charged groups and thus indirectly alter the electrophoretic behavior of the proteins.

The analysis has accounted for approximately 98 per cent of the weight and nitrogen of the intact proteins.

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THE METABOLISM OF GLUCONIC ACID

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In a previous study the appearance of deuterioglucose in the urine of the phlorhizinized rat was shown to follow the injection of *meso*-inositol which had been tagged with deuterium in the carbon-bound positions (1). This result was taken as proof of the glucogenic nature of inositol in the rat and has led the authors to the investigation of another possible glucose precursor, gluconic acid, by a similar approach.

Early studies of the fate of gluconic acid in the intact animal have related chiefly to its oxidation. Thus it has been demonstrated that a large portion of gluconic acid administered is utilized even by the diabetic organism (2-5) and it has been claimed that saccharic acid is one of the oxidation products (3), a claim which has been refuted (6). Several investigators have found that the administration of gluconic acid is followed by the appearance of some unaltered gluconic acid in the urine (5, 6).

Apart from the extensive literature relating to 6-phosphogluconic acid, its formation from glucose-6-phosphate, and its further oxidation and decarboxylation, there are numerous reports of enzyme systems derived from various biological sources capable of oxidizing glucose to gluconic acid, without the demonstrated intervention of a phosphorylated intermediate (7-9). Of particular interest to the present discussion is the glucose dehydrogenase of Harrison (10) isolated from mammalian liver, capable of performing the specific oxidation of glucose to gluconic acid *in vitro* in good yield aerobically in the presence of diphosphopyridine nucleotide (DPN) and methylene blue. The presence of such an enzyme leaves open the possibility that free gluconic acid is formed in the intact liver as a normal oxidative fate of glucose.

In the present experiments doubly labeled gluconic acid has been prepared by the mixing of two samples of sodium gluconate, the one labeled with C^{14} , the other with deuterium. The former was prepared from the starch of bean leaves which had been exposed to $C^{14}O_2$, the latter from the urinary glucose of a diabetic rat receiving D_2O . Such doubly labeled material has been injected into normal and phlorhizinized rats and the distribution of isotopes in expired CO_2 , liver glycogen, urinary gluconate, glucose, and other body constituents has been studied.

EXPERIMENTAL

Preparation of Sodium Gluconate Labeled with C¹⁴ and Deuterium—Starch labeled with C¹⁴ was prepared essentially according to the method of Putman *et al.* (11) with modifications suggested by Gibbs *et al.* (12). Three trifolia were cut from a bush bean plant which had been kept in the dark for 24 hours. These leaves with their stems submerged in water were exposed, in a large desiccator, at slightly less than 1 atmosphere pressure to the CO₂ derived from 500 mg. of BaCO₃ containing 1 mc. of C¹⁴. Two 100 watt incandescent bulbs illuminated the desiccator, which was maintained at 25° for 24 hours. At the end of this period there was no detectable CO₂ in the gas phase. Starch was isolated over the iodine complex; yield, 74 mg. Of this radioactive starch 36 mg. were treated with 2 ml. of *N* HCl at 100° for 3 hours, the resulting solution neutralized with methanolic NaOH, and the glucose oxidized to gluconate by further treatment with I₂ and methanolic NaOH (13).

An adult alloxan-diabetic rat, excreting 4 to 9 gm. of glucose daily, was given 20.9 ml. of an isotonic NaCl solution in 99.8 per cent D₂O in two intraperitoneal injections. The drinking water was replaced by 16 per cent D₂O and during the succeeding 8 days 40 gm. of glucose were excreted in the urine. Half of this urine was concentrated *in vacuo* to a syrup which was then oxidized with I₂ by the procedure of Moore and Link (13) with the sole modification that NaOH replaced KOH. The sodium gluconate was recrystallized from aqueous methanol and it was noted that recrystallization of this compound was more troublesome than that of the potassium salt. 19.3 gm. of recrystallized sodium gluconate were obtained.

5 gm. of deuteriosodium gluconate were added to an aqueous solution of the C¹⁴-sodium gluconate which had been obtained from 36 mg. of C¹⁴-starch, and, after clarification, cautious addition of methanol yielded 3.9 gm. of crystals. One further recrystallization from aqueous methanol gave 3.15 gm. of doubly labeled sodium gluconate; deuterium, 3.49 atom per cent excess; C¹⁴, 160,000 c.p.m. per milliatom of C.

1 gm. of this material was used in the experiment with the phlorhizinized rat, described below. 2 gm. were further mixed in solution with 1.2 gm. of deuteriosodium gluconate, and this was the solution which was subsequently injected into a normal rat. The sodium gluconate in this solution exhibited 102,000 c.p.m. per milliatom of C.

To establish the freedom of this material from radioactive glucose, 20 mg. of the doubly labeled sodium gluconate were dissolved together with 200 mg. of non-isotopic anhydrous glucose in 75 ml. of H₂O. Treatment at 100° for 2 hours with 1.5 ml. of phenylhydrazine and 2 ml. of acetic acid gave a crystalline osazone which, after exhaustive washing with hot water and recrystallization from 60 per cent ethanol, was devoid

of radioactivity. This finding demonstrated that no radioactive glucose contaminated the sodium gluconate preparation. It further showed that no detectable amount of gluconic acid is reduced to glucose under the conditions of osazone formation and that the isolation procedure used is effective in separating glucosazone from all traces of contaminating gluconic phenylhydrazide.

Administration of Isotopic Sodium Gluconate to Normal Rat—The rats employed in the present study were kept in a large vacuum desiccator modified to permit the collection of expired CO_2 . The desiccator was equipped with a coarse wire screen flooring and beneath this a fine wire gauze to separate feces from urine, an inlet and an outlet tube for air, a food jar, and a container for drinking water. Room air was drawn in succession through a flowmeter, a soda-lime tower, the desiccator, and finally through two gas absorption bottles with sintered glass distributing plates. Into the first of these were placed 300 ml., into the second 150 ml. of 7 N NaOH. A few crystals of thymol were placed in the bottom of the desiccator for preservation of the urine. During each experiment a flow of 500 to 600 ml. of air per minute was continuously maintained. The diet, which was offered *ad libitum*, contained 15 per cent casein, 68 per cent corn starch, 5 per cent yeast powder, 4 per cent salt mixture (14), 2 per cent cod liver oil, and 6 per cent refined cotton seed oil. Each rat was acclimated to the desiccator for 1 day prior to the isotope experiment.

An adult male white rat, weighing 227 gm., was given on each of 3 successive days by intraperitoneal injection 3 ml. of a solution containing 1 gm. of doubly labeled sodium gluconate. Prior to injection the pH was adjusted to 7 by addition of HCl. It was estimated that 95 per cent of the material was actually introduced into the animal. The rat appeared normal throughout the experiment except for marked polydipsia and transient evidence of peritoneal irritation manifested by splinting of the hind legs following each injection. On the 3 experimental days the rat consumed approximately 8, 4, and 10 gm. of diet. The desiccator was opened only to permit the daily injection and at these times the 24 hour accumulations of urine and expired CO_2 were collected. After 3 days the rat was killed by a blow on the head; final weight, 212 gm.

The liver, which weighed 9.1 gm., was promptly removed and ground with trichloroacetic acid. Glycogen was precipitated from the trichloroacetic acid solution (15) by the addition of ethanol and was purified by reprecipitation from KOH solution and finally from H_2O (16); yield, 434 mg. The residue from the trichloroacetic extract was defatted with boiling ethanol and ether. The nucleic acids were extracted with hot 10 per cent NaCl solution and their sodium salts precipitated with ethanol

(17). The residue from the NaCl extraction was the material analyzed as "liver protein."

Each urine sample was carefully collected, filtered, and made up to a volume of 100 ml. Aliquots were taken for total C and radioactivity determinations (*cf.* "Analytical methods" below). A solution containing 1 gm. of neutral lead acetate was added to each remaining solution. After 24 hours the mixture was centrifuged and the precipitate washed once with water. Lead was removed from the combined supernatant fluids by treatment with H_2S and the volume reduced *in vacuo* to about 25 ml. 0.5 ml. of phenylhydrazine, 0.3 ml. of acetic acid, and a few crystals of $NaHSO_3$ were added and the mixture heated at 100° under a reflux for 3 hours. The solution was cooled and extracted with ether to remove excess phenylhydrazine. The small amount of precipitate that formed on refrigeration was removed by filtration. The volume of the filtrate was reduced to about 5 ml., and after refrigeration overnight the precipitated yellow crystals were collected by filtration. These were recrystallized from water and colorless needles of gluconic phenylhydrazide were obtained.

$C_{12}H_{18}O_6N_2$. Theory, N 9.8; found, N 10.0

An attempt to isolate saccharic acid as its phenylhydrazide from the insoluble lead salt fraction of the urine was unsuccessful. A carrier method of isolation was therefore employed. The three urine samples from which gluconic phenylhydrazide had been isolated were combined and 100 mg. of non-isotopic saccharic phenylhydrazide were added. From a solution of the mixture saccharic phenylhydrazide was isolated and three times recrystallized from large volumes of aqueous ethanol. To insure the absence of contaminating radioactive gluconic phenylhydrazide in this material, 50 mg. of it were dissolved together with 50 mg. of non-isotopic gluconic phenylhydrazide and the saccharic acid derivative reisolated and recrystallized. The specific activity of saccharic phenylhydrazide was as follows: twice recrystallized, 47 c.p.m. per milliatom of C; three times recrystallized, 51 c.p.m. per milliatom of C; and "washed out" with gluconic phenylhydrazide, 48 c.p.m. per milliatom of C.

The freedom of the injected sodium gluconate from contaminating radioactive saccharic acid was established by a similar "washing out" procedure in which non-isotopic saccharic acid was added to and reisolated from a solution of the radioactive gluconic acid.

A preliminary exploration of the fate of gluconic acid in the rat was carried out by the same methods. The material injected was sodium gluconate exhibiting 12,800 c.p.m. per milliatom of C, and the study of

the animal was somewhat less complete than in the experiment described above. The pertinent results from this study are included in the present report.

Administration of Isotopic Sodium Gluconate to Phlorhizinized Rat—An adult male white rat, weighing 232 gm., was injected subcutaneously with 0.25 ml. of a 10 per cent suspension of phlorhizin in sesame oil. A similar preliminary injection had resulted in an excretion of 0.75 gm. of glucose in 17 hours. No food was given for 3 hours previous to the phlorhizin injection nor during the course of the experiment. 1 gm. of sodium gluconate (160,000 c.p.m. per milliatom of C), dissolved in 6 ml. of H_2O and adjusted to pH 7 with HCl, was injected intraperitoneally 2 hours after administration of the phlorhizin. During the next 24 hours the rat was kept in the desiccator equipped as previously described and expired CO_2 and excreted urine were collected. The rat was stuporous for several hours after the injection, had marked dyspnea, and slept for most of the remaining time. At the end of the 24 hour period the rat was killed by a blow on the head and the liver quickly removed and dissolved in hot 30 per cent aqueous KOH. No glycogen could be isolated. C and C^{14} analyses were carried out on aliquots of the NaOH solution in which the expired CO_2 had been collected. 0.326 mole of CO_2 exhibiting 1400 c.p.m. per milliatom of C was expired in 24 hours.

The small amount of voided urine and that remaining in the bladder were combined and glucose isolated as its osazone. The osazone was twice extracted with 50 ml. of boiling water and filtered from the hot solution. This procedure had previously been shown to remove all traces of gluconic phenylhydrazide from glucosazone. The resulting 201 mg. of osazone were recrystallized first from 60 per cent ethanol and then from pyridine-benzene without change in radioactivity. The yield of twice recrystallized glucosazone was 125 mg.

Degradation and Allocation Studies—Glucosazone was oxidized with periodate by a combination of methods described by others (18–20). 36 mg. of recrystallized glucosazone from the urine of the phlorhizinized rat were dissolved in 15 ml. of hot 66 per cent ethanol and the solution was cooled to 30° and treated with 1 ml. each of 1 N NaOH and 0.3 M periodic acid. After 15 minutes the precipitated 1,2-bisphenylhydrazone of mesoxaldehyde was removed by centrifugation and recrystallized from 66 per cent ethanol. The supernatant was promptly acidified with HCl and freed of excess periodate by addition of 1 ml. of 1.2 N Na_2HAsO_4 . The solution was made alkaline and formaldehyde distilled in a stream of N_2 into a chilled receiver. Formaldehyde was precipitated as its dimedon derivative (18). The formic acid in the undistilled residue was

oxidized by treatment with HgO in acid solution and the CO_2 collected in $\text{Ba}(\text{OH})_2$ solution. From the analyses of these derivatives the radioactivity in C-(1 + 2 + 3), in C-(4 + 5), and in C-6 was calculated.

A portion of the liver glycogen from the normal rat was converted to glucosazone, which was degraded and analyzed in the same way, giving the distribution of radioactivity in C-(1 + 2 + 3) in C-(4 + 5), and in C-6. Another portion of liver glycogen was hydrolyzed and oxidized directly to give values for C-(1 + 2 + 3 + 4 + 5) and C-6.

Analytical Methods—Aliquots of alkaline solutions which contained radioactive CO_2 were transferred to the Van Slyke manometric apparatus, where the CO_2 was liberated and measured. All organic compounds to be analyzed for C^{14} were oxidized by the wet ash procedure of Van Slyke and Folch (21) and the CO_2 collected in the pipette of the manometric apparatus. Aliquots of materials such as urine were evaporated to dryness in combustion tubes of the type described by the above authors and the residues were oxidized by the wet ash procedure. The CO_2 , which at the termination of this manometric analysis was in the gas phase, was then transferred to a 15 ml. centrifuge tube containing 5 ml. of a solution of approximately 0.25 N $\text{Ba}(\text{OH})_2$ in 2 per cent of BaCl_2 (22). The transfer was effected through a short piece of capillary tubing connected to the side arm of the Van Slyke apparatus and fitted with a 1-hole rubber stopper which accommodated the centrifuge tube. The precipitated BaCO_3 was twice washed with CO_2 -free water by centrifugation with protection against contaminating atmospheric CO_2 and finally suspended in water.

The BaCO_3 was plated for counting by filtration on a disk of scintered alundum of medium porosity. Disks nominally $1 \times 1/8$ inches¹ were ground to flatness and uniform thickness by rubbing their wet surfaces against each other. These were mounted under glass chimneys constructed of precision bore tubing of 0.750 inch internal diameter with ground flat ends. The BaCO_3 suspension in ample water was poured into the chimney and gentle suction applied. The precipitate was sucked dry, the chimney carefully removed, and the disk placed upon clean aluminum foil on an electric hot-plate which was equipped with a thermometer. The temperature was slowly allowed to rise to 160° (23).

Planchets were uniformly mounted beneath a thin mica window Geiger-Müller tube. A standard sample of $\text{BaC}^{14}\text{O}_3$, similarly mounted, was frequently counted and all analyses were normalized with reference to this sample. In addition to the conventional corrections for background, self-absorption, and coincidence error, an additional correction for the

¹ E. Machlett and Son, No. 34-230-B.

contaminating non-radioactive carbon, based on the value of the blank c in the manometric analysis, was applied.

The weight of the sample of BaCO_3 was determined at the end of the counting operation by weighing the alundum disk before and after dissolving the precipitate in dilute HCl . No significant residual radioactivity could be detected at the end of an analysis in the combustion tube, in the Van Slyke pipette, or in the cleaned planchet. With the procedures and equipment described, "infinite thickness" of BaCO_3 was found to have been reached when the precipitate weighed 34 mg. Whenever possible, "infinitely thick" samples were counted.

All analytical values are expressed as specific activity which is defined for the purpose of the present communication as counts per minute per milliatom of C.

DISCUSSION

The sodium gluconate administered in the present study was a mixture of the products derived from the oxidation of two biosynthetic sugars, deuterioglucose excreted by a diabetic rat and C^{14} -starch photosynthesized by bean leaves. Glucose excreted by a diabetic animal receiving D_2O has previously been shown to be approximately uniformly labeled in all its stable positions (24) and starch accumulating in a leaf after prolonged exposure to C^{14}O_2 has likewise been reported to be uniformly labeled (25), a finding confirmed in this laboratory. The sodium gluconate administered was therefore approximately uniformly labeled with respect to both isotopes.

The results obtained from the experiment in which isotopic sodium gluconate was injected intraperitoneally into a normal rat for three successive days are given in Table I. It will be seen that, of the C^{14} administered as gluconate, 57 per cent was promptly excreted in the urine while 14 per cent appeared in the expired CO_2 . Thus, of the 43 per cent which was not lost in the urine, approximately a third appeared as respiratory CO_2 . This finding gives some idea of the extent to which gluconic acid is catabolized and indicates furthermore that the observed oxidation involves more than mere decarboxylation to pentose. If only C-1 were contributing to CO_2 , no more than a sixth of the carbon of retained gluconate would have been recoverable in this fraction.

Several of the components of the liver were found to contain C^{14} . It is noteworthy that the nucleic acids were almost as rich in isotope as was the glycogen. Because of the shortage of material no attempt has been made to determine the specific activity of nucleic acid pentose, but the present finding is compatible with the suggestion that gluconic or phosphogluconic acid (26, 27) may serve as a precursor of the 5-carbon sugars.

From the urine excreted on each of the 3 days the phenylhydrazide of gluconic acid was isolated in the pure state and analyzed for both C^{14} and D. When these values are compared with the corresponding values obtained on the material administered, it is apparent that, within the limits of the analytical methods, neither isotope has undergone any dilution incident to the passage of gluconate through the body. Had the tissues of the rat contributed as little as 50 mg. of gluconic acid per day to the material which was presented to the kidney for excretion, a dilution of

TABLE I

Distribution of Isotopes after Administration of Doubly Labeled Sodium Gluconate to Normal Rat for 3 Days

Sample analyzed	C^{14} specific activity	Total carbon	Total radioactivity, (a) $\times$ (b) = (c)	Per cent dose administered 100 (c)	Deuterium excess
	(a)	(b)		8.0×10^4	
	<i>c.p.m. per milliatom C</i>	<i>milliatoms</i>	<i>c.p.m.</i>	<i>per cent</i>	<i>atom per cent</i>
Na gluconate injected.	102,000	78.5	8.0×10^4	100	3.49*
Urine C, Day 1.	66,600	28.2	1.88×10^4		
“ “ “ 2.	46,900	20.2	0.95×10^4	57	
“ “ “ 3.	61,200	27.6	1.69×10^4		
Expired CO_2 , Day 1.	757	292	0.221×10^4		
“ “ “ 2.	1,790	306	0.548×10^4	14	
“ “ “ 3.	1,100	292	0.322×10^4		
Liver, glycogen.	492	16.1	0.008×10^4	0.1	
“ nucleic acids.	468				
“ “proteins”	305				
Gluconate from urine, Day 1. . .	104,000†				3.54*
“ “ “ “ 2.	101,000†				3.54*
“ “ “ “ 3.	102,000†				3.50*

* Calculated from analyses on phenylhydrazides by multiplication by 18/11.

† Calculated from analyses on phenylhydrazides by multiplication by 12/6.

both isotopes in the urinary product would at once have been apparent and these results are therefore taken to mean that free gluconic acid is formed very little if at all in the tissues of the normal rat. This conclusion is not to be construed as in any way affecting the possibility of the formation of phosphogluconic acid from hexose phosphate (28), since, in the absence of an appropriate phosphatase, phosphogluconic acid would not mix with gluconic acid. It would appear, however, that the oxidation of glucose to free gluconic acid observed to be catalyzed *in vitro* by glucose dehydrogenase prepared from normal liver does not, in the intact animal, make any detectable contribution to gluconic acid in the blood.

The results of a preliminary experiment carried out in similar fashion, except that a much smaller quantity of C^{14} was employed, are given in Table II. The values obtained in this experiment were essentially in agreement with those just discussed and again it will be noted that the specific activity of the urinary gluconate was the same as that of the sodium gluconate injected.

As in the normal rat, so in the phlorhizinized rat, when isotopic sodium gluconate was injected, the expired CO_2 was found to have about 1 per cent of the specific activity of the material injected (Table III) and represented about 10 per cent of the total dose of C^{14} . Of particular interest in this case was the urinary glucose, the carbon of which proved

TABLE II

Distribution of Isotopes after Administration of Labeled Sodium Gluconate to Normal Rat for 3 Days

Sample analyzed	C^{14} specific activity (a)	Total carbon (b)	Total radioactivity, (a) $\times$ (b) = (c)	Per cent dose administered $\frac{100(c)}{10.0 \times 10^5}$
	<i>c.p.m. per million C</i>	<i>milliatoms</i>	<i>c.p.m.</i>	<i>per cent</i>
Na gluconate injected	12,800	78.5	10.0×10^5	
Urine C	6,870	77.0	5.28×10^5	53
Expired CO_2 , Day 1	152	248	0.377×10^5	
" " 2	137	334	0.458×10^5	
" " 3	238	280	0.666×10^5	15
Liver, glycogen	51	17.2	0.009×10^5	0.1
" nucleic acids	66			
Gluconate from urine	12,500			

to be about twice as radioactive as was the carbon of the CO_2 . Whereas this finding in itself made it unlikely that the radioactivity of the glucose had arisen exclusively from tissue CO_2 , it was deemed advisable to study the distribution of C^{14} among the 6 carbon atoms of this sample of glucose, the fact having been well established that CO_2 contributes exclusively to C-3 and C-4 of glucose in the body of the rat (29). The fragments analyzed represented carbon atoms 1, 2, and 3, atoms 4 and 5, and atom 6. As will be seen in Table III, the specific activities of each of these three fragments were of the same order of magnitude and indicated an approximately uniform distribution of C^{14} among the 6 carbon atoms of the excreted glucose. A similar degradation of the sample of liver glycogen obtained from the normal rat receiving isotopic sodium gluconate also revealed similar uniformity of specific activity of the frag-

ments (Table IV). These findings, considered together with the fact that the gluconate administered was also uniformly labeled with C^{14} , demonstrate clearly the occurrence of a transformation of gluconic acid into glucose *in vivo* which proceeds in some fairly direct fashion.

The question of the possible conversion of gluconic acid to saccharic acid and its subsequent excretion in the urine was explored by the addition of 108 mg. of non-isotopic carrier saccharic acid, as its phenylhydrazide, to the combined urine fractions from which gluconic

TABLE III

Allocation of C^{14} in Urinary Glucose

A phlorhizinized rat was given 1 gm. of isotopic sodium gluconate for 1 day.

	C^{14} specific activity c.p.m. per milliatom C
Na gluconate injected.	160,000
Expired CO_2	1,400
Urinary glucose	2,770
C-(1 + 2 + 3)	3,430
C-(4 + 5)	2,510
C-6	2,380

TABLE IV

Allocation of C^{14} in Liver Glycogen from Normal Rat Given Isotopic Sodium for 3 Days

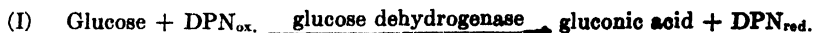
The glycogen was that obtained from the experiment described in Table I.

Source of carbon	C^{14} specific activity c.p.m. per milliatom C
Liver glycogen	492
C-(1 + 2 + 3 + 4 + 5)	420
C-(1 + 2 + 3)	455
C-(4 + 5)	383
C-6	560

phenylhydrazide had been removed. This material was subsequently reisolated, recrystallized to constant radioactivity, and then "washed out" to insure the absence of contaminating radioactive gluconic acid. Assuming any urinary saccharate to have the same specific activity as that of the gluconate, 102,000 c.p.m. per milliatom, and computing the activity of the finally isolated saccharate from that of its phenylhydrazide, $48 \times (18/6) = 144$ c.p.m. per milliatom, $(108 \times 144)/(102,000 - 144) = 0.15$ mg. of saccharic acid is all that can be accounted for as having appeared in the urine. This number, when compared with 3000 mg. of

sodium gluconate injected, reveals that only about 0.005 per cent of the injected gluconate, a negligible fraction, is recoverable as urinary saccharate.

The results, considered together, would make it appear that the reaction



certainly contributes very little if any gluconic acid to the fluids of the body, despite the demonstrated presence of the enzyme in the liver. It is clear that gluconic acid is oxidized in the animal body, but present evidence affords no support to the view that saccharic acid is an important intermediate. The transformation of gluconic acid to glucose which was found to occur has been shown not to proceed predominantly over CO_2 . Alternative hypotheses to account for the data are the direct reduction of the acid to the aldehyde, or the degradation or fragmentation of gluconic acid to 3-carbon fragments and subsequent resynthesis of hexose. It seems unlikely that a reduction of gluconic acid by simple reversal of Reaction I can occur to any appreciable extent in view of the exergonic nature of the reaction as written.² Some reductant more potent than $\text{DPN}_{\text{red.}}$ may, however, participate in the observed transformation.

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SUMMARY

Sodium gluconate uniformly labeled with C^{14} and D has been prepared and administered to normal and phlorhizinized rats. The occurrence of extensive biological oxidation of gluconate has been confirmed by the presence of C^{14}O_2 in the expired air.

A considerable quantity of gluconic acid was excreted in the urine and was found to be isotopically identical with that injected. The failure of the tissues to contribute gluconic acid *in vivo* contrasts with the occurrence *in vitro* of a liver enzyme capable of converting glucose into free gluconic acid.

Only a negligible fraction of gluconate carbon could be recovered as urinary saccharate. Both nucleic acid and glycogen isolated from the liver of the normal rat exhibited significant radioactivity.

The urinary glucose from the phlorhizinized rat and the liver glycogen from the normal rat were found to be approximately uniformly labeled

² From published values of E' 's (pH 7.0) for glucose to gluconate and for coenzyme I (30), the equilibrium constant for the reaction as written has been calculated as approximately 5×10^4 and ΔF as -8000 calories per mole.

with respect to C¹⁴. This indicates a more or less direct conversion of gluconic acid into glucose.

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STUDIES ON THE MECHANISM OF NITROGEN STORAGE

IV. MUSCLE TRANSAMINASE, FREE AMINO ACIDS, AND GLUTAMINE IN NORMAL AND INDUCED STATES OF GROWTH

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An important function for transamination in the dynamic state of amino nitrogen has been suggested in a number of studies in protein metabolism. Particularly significant is the classical work of Schoenheimer *et al.* (1) in which the highest uptake of ingested N^{15} was found to occur in the dicarboxylic acids of tissue proteins and especially in glutamic acid. Of considerable interest in this connection is the inverse relationship between transamination and the synthesis of protein suggested by Cohen and Hekhuis (2). They observed low transaminase activity in rapidly growing embryonic, malignant, and regenerating tissue. A rôle for transamination in the biological synthesis of peptide intermediates, proposed by Linderstrøm-Lang (3), has also been considered by Herbst and Shemin (4) in connection with reactions involving transamination of α -ketoacylamino acids with glutamic acid.

Since nitrogen storage induced in animals by the administration of growth hormone is characterized by protein anabolism, it seemed of interest in continuing our work on the mechanism of this effect (5-7) to examine the process of transamination in tissues obtained from rats in which growth had been arrested by hypophysectomy or had been induced by the administration of anterior pituitary growth hormone. Parallel studies on the total amino acid carboxyl nitrogen and glutamine carboxyl nitrogen were also of interest, since in earlier work we had found marked changes in both the plasma amino acid and glutamine concentrations in induced states of growth (5).

EXPERIMENTAL

Animals—Female rats of the Sprague-Dawley strain, both normal and hypophysectomized, were used in all the experiments. The hypophysectomized animals¹ were 26 to 28 days old at the time of operation and usually required about 2 postoperative weeks to reach a plateau in body weight. Normal female rats 5 to 6 months old were used in some of the studies.

¹ Purchased from the Hormone Assay Laboratories of Chicago, Illinois.

Diets—The animals were maintained on a diet (6) containing 24 per cent casein previously shown to be optimal for the production of nitrogen storage in hypophysectomized rats (8).

*Growth Hormone Preparations*²—Preparations assayed by means of gain in weight of normal or hypophysectomized rats and, in some of the studies, by both nitrogen storage and gain in weight in adult female dogs were used in all the experiments.

Methods—The lyophilized preparations of growth hormone were prepared for injection by the intraperitoneal route as previously described (6). In general, the experiments in induced growth on hypophysectomized and normal adult female rats consisted of a 5 day control period during

TABLE I
Skeletal Muscle Transaminase of Normal Adult Female Rats

No. of rats	Feeding method and treatment	Change in body weight	Transaminase activity, O_T^{10}	
		gm.	per mg. dry weight	per mg. homogenate total N
6	<i>Ad libitum</i> ; no treatment	+3.3	+42.9	+317
9*	Pair-fed with untreated; 5-15 mg. growth hormone Preparation Rx099916	+15.3	+42.4	+313

Preparation Rx099916 (Parke, Davis and Company) assayed at 3502 units per gm. in normal adult female rats. Assays in hypophysectomized rats were equivalent to about 91 per cent of that obtained at the 10 γ per day level for a 10 day injection period with pure crystalline growth hormone described by Fishman, Wilhelmi, and Russell (13).

* Since the response of three groups of rats (three rats per group) treated respectively with 5, 10, and 15 mg. of Preparation Rx099916 was practically the same in respect to gain in body weight, the data are recorded as the average for nine animals.

which it was ascertained that the animal had reached a plateau in body weight, followed by a 10 day period in which growth was induced in the animal by administration of the anterior pituitary growth hormone preparation. The rats were sacrificed by a sharp blow on the head and decapitation. The hind legs of the animals were quickly skinned, disarticulated at the hip, and stored frozen in dry ice until assayed. Tibialis anticus muscle was removed in the frozen state and homogenates were prepared by freezing the tissue in liquid nitrogen, pulverizing in a steel mortar, and homogenizing by the Potter-Elvehjem technique (9). Determinations of

² We are especially indebted to Dr. D. A. McGinty and Mr. L. W. Donaldson of Parke, Davis and Company, and to Dr. John R. Mote and Mr. I. Bunding of the Armour Laboratories for supplying assayed preparations.

aspartic-glutamic transaminase were conducted according to the method of Ames and Elvehjem (10). Transaminase activities expressed in terms of Q_T^{10} are the average of duplicate determinations conducted on tibialis

TABLE II

Skeletal Muscle Transaminase, Total Amino Acid Carboxyl Nitrogen, and Glutamine Carboxyl Nitrogen in Normal and Induced States of Growth

Female rats* (5 rats per group)	Feeding method and treatment	Change in body weight	Transaminase activity, Q_T^{10}			Carboxyl nitrogen		
						Total (1)	Glutamine (2)	Per cent of (2) in (1)
		gm.	per mg. homogenate total N†	per gm. wet tissue	per 100 gm. body weight‡	mg. per cent	mg. per cent	
Hypophysectomized..	<i>Ad libitum</i> ; no treatment	+1.2	+297 (±8)	+8590	+3230	24.3	8.2	33.7
"	Pair-fed with untreated; 275 γ Preparation 22KR2§	+14.4	+247	+7040	+2610	28.3	2.2	7.6
"	Pair-fed with untreated; 600 γ Preparation 22KR2	+16.0	+249 (±11)	+7200	+2710	30.8	4.9	15.9
"	Pair-fed with untreated; 1 mg. Preparation 22KR2	+16.4	+242	+7120	+2725	27.2	4.3	15.8
Normals....	Pair-fed with untreated; no treatment	+5.9	+275	+8200	+3510	25.5	5.7	22.4
"	<i>Ad libitum</i> ; no treatment	74.8	+229	+8000	+1960	29.1	6.4	22.0

* The normal animals were of the same age group as the hypophysectomized animals (26 to 28 days of age).

† The values in parentheses are standard deviations from the mean.

‡ Calculated according to Miller (14).

§ Preparation 22KR2 (Armour Laboratories), reported to be 95 per cent one component by electrophoretic analysis, was particularly potent in the production of nitrogen storage and gain in body weight in the adult female dog (unpublished data). The total treatment indicated was spread over a 10 day injection period.

anticus homogenates prepared from each rat. Total amino acid carboxyl nitrogen, *i.e.* nitrogen calculated as 1 gm. atom of nitrogen per mole of carbon dioxide evolved by the reaction of ninhydrin with α -amino acids, and glutamine carboxyl nitrogen were determined by the method of

Hamilton (11). Determinations were conducted in duplicate on pooled leg muscle obtained from two to three rats. Total amino acid carboxyl nitrogen reported for tissue in our studies is approximately 10 per cent low, due to small losses occurring during the preliminary removal of glutathione and ascorbic acid. Determinations of total nitrogen in tissue homogenate were run in duplicate according to the conditions of Hiller, Plazin, and Van Slyke (12) with titrimetric determination of ammonia.

TABLE III

Transamination, Total Amino Acid Carboxyl Nitrogen, and Glutamine Carboxyl Nitrogen in Normal and Hypophysectomized Rats and Hypophysectomized Rats Treated with Anterior Pituitary Growth Hormone

Female rats (6 rats per group)	Feeding method and treatment	Change in body weight	Transaminase activity, Q_T^B			Carboxyl nitrogen		
						Total (1)	Glutamine (2)	Per cent of (2) in (1)
		gm.	per mg. homogenate total N	per mg. dry tissue	per gm. wet tissue	mg. per cent	mg. per cent	
Hypophysectomized..	<i>Ad libitum</i> ; no treatment	+3.3	+289 (± 22)	+49.3	+8733	25.7	6.5	25.5
" ..	Pair-fed with untreated; 250 γ Preparation Rx099916*	+10.4	+263	+40.2	+7283	28.7	4.5	15.4
" ..	Pair-fed with untreated; 500 γ Preparation Rx099916	+15.0	+245 (± 19)	+33.0	+7150	27.4	5.1	18.7
Normals.....	Pair-fed with untreated; no treatment	+5.8	+308	+42.6	+9066	25.4	4.9	19.0

* Preparation Rx099916 described in foot-note in Table I.

Results

In Table I are recorded the results of studies on muscle transaminase of normal adult female rats. Although the animals which received the preparation of anterior pituitary growth showed an increase in body weight, no significant changes were observed in transaminase activity. Determinations of total carboxyl nitrogen on pooled leg muscle from controls fed *ad libitum* and the pair-fed treated animals resulted in values of 20.3 and 18.0 mg. per cent respectively. Values of glutamine carboxyl nitrogen were 4 and 3.7 mg. per cent respectively. Glutamine carboxyl nitrogen constituted 19.5 per cent of the total carboxyl nitrogen in the controls and 20.5 per cent in the treated animals.

In Table II are summarized the results of a study on 26 to 28 day-old female rats. Tibialis anticus homogenates prepared from hypophysectomized rats treated with anterior pituitary growth hormone and from rapidly growing untreated animals showed uniformly lower Q_r^{10} values than were observed in similar homogenates prepared from hypophysectomized rats fed *ad libitum* or from pair-fed normal rats. Total amino acid carboxyl nitrogen in pooled leg muscle obtained from pair-fed hypophysectomized animals treated with growth hormone is from 12 to 25 per cent above that found for untreated hypophysectomized controls. The average total amino acid carboxyl nitrogen for the treated animals closely approaches that of rapidly growing normal rats fed *ad libitum*. Glutamine carboxyl nitrogen concentrations were observed to drop from 8.2 mg. per cent in the untreated hypophysectomized controls to an average of 3.8 mg. per cent in the pair-fed treated hypophysectomized animals. A marked drop in the percentage of glutamine carboxyl nitrogen in the total carboxyl nitrogen in skeletal muscle was observed in the hypophysectomized rats treated with growth hormone. Table III shows the results of a similar study in which the rats were treated with the growth hormone preparation used in the experiments described in Table I. The results confirm those presented in Table II.

The effect of different concentrations of growth hormone *in vitro* on the transaminating capacity of homogenates of tibialis anticus prepared from untreated hypophysectomized animals was also tested. Concentrations from 0.1 γ to 1 mg. of growth hormone were added directly to Warburg flasks containing homogenate equivalent to 5 mg. of wet tissue. Transamination was unaffected.

DISCUSSION

The results of our studies on concentrations of total amino acid and glutamine carboxyl nitrogen in skeletal muscle in states of growth arrested by hypophysectomy or induced by the administration of growth hormone and in normal adult animals at a growth plateau are in complete agreement with the concept that growth hormone may produce some of its effects through diminished amino acid catabolism. In comparison with skeletal muscle transaminase in normal adult female rats and in rats in which growth has been arrested by hypophysectomy, we have found a relatively low activity in tibialis anticus homogenates prepared from rapidly growing normal rats and from hypophysectomized rats in which growth was induced with anterior pituitary growth hormone. Examination of the Q_r^{10} data expressed on the basis of homogenate total nitrogen suggests that the low transaminase activity might be due to increased homogenate total nitrogen. In this respect, our nitrogen data show the changes in transaminase to be very real ones. The average total nitrogen in homogenate for both the

hypophysectomized untreated rats fed *ad libitum* and the hypophysectomized rats treated with growth hormone at the three different dosages shown in Table III was 735 γ per ml. of 2.5 per cent homogenate. Statistical analysis (15) of transaminase data (Table II) expressed on the nitrogen basis for the untreated hypophysectomized rats fed *ad libitum* and the hypophysectomized rats treated with 600 γ of growth hormone Preparation 22KR2 showed the difference to be highly significant. A P value <0.01 was obtained. Similarly, in Table III, the difference in transaminase activity between untreated hypophysectomized controls and pair-fed hypophysectomized rats treated with 500 γ of growth hormone was found to be highly significant. Total amino acid carboxyl nitrogen in the skeletal muscle of hypophysectomized rats treated with growth hormone and in muscle of rapidly growing normal rats is higher than in normal adult and untreated hypophysectomized rats. Glutamine carboxyl nitrogen in muscle in states of induced growth and in rapidly growing normal animals is lower than in states of growth arrested by hypophysectomy.

Utilizing isotopes, Hoberman (16) has recently published the results of an extensive study on the endocrine regulation of amino acid and protein metabolism during fasting. In this work the rate-process constants for the protein $\rightleftharpoons$ amino acid equilibrium and for the catabolism of amino acids to urea were evaluated from N^{15} excretion. Hypophysectomy resulted in an increase in the rate-process constant for amino acid catabolism and treatment of hypophysectomized rats with growth hormone returned the constant to normal. Synthesis of protein from amino acids was shown to be only slightly affected by hypophysectomy, but definitely accelerated in the hypophysectomized animal treated with anterior pituitary growth hormone. Data in terms of the rate-process constants for the over-all reactions thus appear to be in complete agreement with our observations on total free amino acid and glutamine concentrations.

Since we observed low skeletal muscle transaminase in induced states of growth and in rapidly growing normal animals, studies on the magnitude of distribution ratios of amino acids between cells and extracellular fluid following the feeding of individual amino acids are of considerable interest. Christensen and coworkers (17) found that feeding glutamic acid resulted in an increase in the distribution ratio of other amino acids between the cells and the extracellular fluid. Unfortunately, our study did not include determinations of the concentrations of amino acids in the extracellular compartment. Li *et al.* (18) have reported, however, a reduction of plasma amino acids in normal rats treated with anterior pituitary growth hormone. Our observation of a low transaminase activity in skeletal muscle in induced states of growth and in rapidly growing normal animals may be indicative of a mechanism initiated by growth hormone providing for the

conservation of glutamic acid and the subsequent concentration of amino acids promoting nitrogen storage and the intracellular synthesis of protein.

SUMMARY

1. Transaminase activity in tibialis anticus muscle of rapidly growing normal rats is lower than in similar tissue of adult normal and immature hypophysectomized rats.

2. Growth hormone preparations at low dosage levels, sufficient to produce the physiological response of gain in body weight, produced a significant decrease in the transaminase activity of tibialis anticus muscle of immature hypophysectomized rats.

3. Concentrations of total amino acid carboxyl nitrogen in skeletal muscle of hypophysectomized rats in induced states of growth and in rapidly growing normal rats are higher than in normal adult and untreated hypophysectomized rats.

4. Concentrations of glutamine carboxyl nitrogen in skeletal muscle are lower in hypophysectomized rats in induced states of growth and in rapidly growing normal rats than in rats in which growth has been arrested by hypophysectomy.

5. Preparations of anterior pituitary growth hormone produced no effect *in vitro* on the transaminating capacity of homogenates prepared from tibialis anticus muscle of hypophysectomized rats.

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STUDIES ON THE MECHANISM OF NITROGEN STORAGE

V. HEPATIC AND RENAL TRANSAMINASE IN NORMAL AND INDUCED STATES OF GROWTH

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Increased amino acid and decreased glutamine concentrations in skeletal muscle of rapidly growing normal rats and in skeletal muscle of hypophysectomized rats in induced states of growth (1) are in agreement with the concept that growth hormone may produce some of its effects through diminished amino acid catabolism. In considering mechanisms for the oxidative deamination of L-amino acids, Braunstein and Bychkov (2) proposed a two-step process whereby the amino acids were first transaminated with α -ketoglutaric acid and the resulting glutamic acid then deaminated by glutamic dehydrogenase. Braunstein and Azarkh (3, 4) actually demonstrated the deamination of certain amino acids by this mechanism in surviving liver and kidney tissue. Since diminished amino acid catabolism might be accompanied by changes in the transaminating capacity of liver and kidney tissues, it seemed of interest to include hepatic and renal transaminase in our studies on the mechanism of nitrogen storage.

EXPERIMENTAL

The liver and kidney tissues used in the transamination studies were obtained from animals sacrificed during the course of the experiments reported in the preceding paper (1), in which details concerning diets and treatment are given. Hepatic and renal transaminase were determined according to the method of Ames and Elvehjem (5). The results are expressed in terms of Q_T^{10} on the basis of tissue dry weight.

Results

In Table I are recorded the results of studies on normal adult female rats. Production of the physiological response of gain in body weight in the adult rat treated with anterior pituitary growth hormone and pair-fed with untreated controls was unaccompanied by changes in either hepatic or renal transaminase.

Table II summarizes the data obtained from studies in immature female rats. Hypophysectomy appears to have no effect on the hepatic

TABLE I

Effects of Anterior Pituitary Growth Hormone on Hepatic and Renal Transaminase of Normal Adult Rats

No. of rats*	Feeding method and treatment	Change in body weight	Liver		Kidneys	
			Total weight	Q_T^{10}	Total weight	
		gm.	gm.		gm.	
6	<i>Ad libitum</i> fed; no treatment	-3.3	5.94	+24.4	1.71	+29.1
9†	Pair-fed with untreated; 5-15 mg. Preparation Rx099916‡	+15.3	5.64	+24.7	1.61	+28.9

* Adult female rats, 5 to 6 months old.

† The gain in body weight of three groups of rats (three rats per group) treated respectively with 5, 10, and 15 mg. of Preparation Rx099916 varied only from 14.4 to 16.0 gm. The data are, therefore, recorded as the average for the nine animals.

‡ Growth hormone Preparation Rx099916, previously described (1).

TABLE II

Hepatic and Renal Transaminase in Normal and Hypophysectomized Rats and in Hypophysectomized Rats Treated with Anterior Pituitary Growth Hormone

Group No. (5 rats per group)	Description*	Feeding method and treatment	Change in body weight	Liver		Kidneys	
				Total weight	Q_T^{10}	Total weight	Q_T^{10}
			gm.	gm.		gm.	
I	Hypophysectomized	<i>Ad libitum</i> fed; no treatment	+4.1	2.834	+28.6 (± 3.3)†	0.528	+31.4
II	"	Pair-fed with Group I; treated with 275 γ Preparation 22KR2‡	+14.4	2.381	+32.9 (± 5.0)	0.542	+38.1
III	"	Pair-fed with Group I; treated with 600 γ Preparation 22KR2	+16.0	2.446	+42.2 (± 6.9)	0.543	+30.9
IV	"	Pair-fed with Group I; treated with 1 mg. Preparation 22KR2	+21.5	2.497	+57.4 (± 14.4)	0.611	+29.3
V	Normals	Pair-fed with Group I; no treatment	+5.9	2.474	+42.0 (± 11.4)	0.713	+36.2
VI	"	<i>Ad libitum</i> fed; no treatment	+74.8	6.789	+25.9 (± 7.0)	1.358	+29.8

* The hypophysectomized rats were 26 to 28 days old at the time of operation. Normal rats were from the same age group.

† Growth hormone Preparation 22KR2, previously described (1).

‡ The values in parentheses are the standard deviations calculated according to Fisher (6).

and renal transaminase of the rapidly growing normal rat fed *ad libitum* (Groups I and VI). A decrease in liver weight and an increase in hepatic transaminase were observed in pair-fed hypophysectomized rats treated with growth hormone (Groups II, III, and IV) and in normal rats pair-fed against them (Group V). The drop in total liver weight observed in the pair-fed hypophysectomized rats occurred in spite of the over-all gain in body weight following administration of growth hormone. Although kidney weights were slightly higher in the treated animals, renal transaminase was not significantly altered. Liver and kidneys of normal rats, pair-fed with untreated hypophysectomized controls, followed a similar weight pattern. Differences in hepatic transaminase between Groups I and III and Groups I and IV were found to be highly significant by the

TABLE III
Hepatic and Renal Transaminase in Induced States of Growth

Group No. (6 rats per group)	Description*	Feeding method and treatment	Change in body weight	Liver		Kidneys	
				Total weight	Q_T^{10}	Total weight	Q_T^{10}
			gm.	gm.		gm.	
I	Hypophysectomized	<i>Ad libitum</i> fed; no treatment	+1.3	2.448	+37.7	0.452	+35.0
II	"	Pair-fed with Group I; treated with 50 γ Preparation Rx099916 per day for 10 days†	+15.0	2.330	+43.6	0.558	+35.3

* Hypophysectomized rats were 26 to 28 days old at the time of operation.

† Growth hormone Preparation Rx099916, previously described (1).

methods of Fisher (6). *P* values <0.01 were obtained. Differences in hepatic transaminase between Groups I and V and Groups V and VI resulted in *P* values of approximately 0.05 and cannot be considered significant.

In Table III are summarized the results of similar studies on another series of hypophysectomized rats. These experiments differ from those recorded in Table II only in respect to the growth hormone preparation used in the induction of growth. Preparation Rx099916, described in Table I, was administered at the 50 γ per day level for a period of 10 days. Changes in hepatic and renal transaminase and changes in body weight and organ weights confirm the general pattern shown by data recorded in Table II.

Experiments *in vitro* in which growth hormone was added directly to

Warburg flasks containing liver or kidney homogenates prepared from untreated hypophysectomized rats were negative. Addition of 10 γ and 100 γ quantities of growth hormone (lot No. 22KR2) to flasks containing homogenates equivalent to 2.5 to 12.5 mg. of wet tissue produced no change in the Q_T^{10} value.

DISCUSSION

The parallel observations of loss in liver weight, increase in body weight, and elevated hepatic transaminase in hypophysectomized rats treated with anterior pituitary growth hormone and pair-fed with untreated hypophysectomized controls fed *ad libitum* are particularly interesting. The pair-fed hypophysectomized rat in a greatly stimulated state of growth, induced through the administration of anterior pituitary growth hormone, appears to be unable to secure adequate supplies of essential structural components from its limited dietary intake for anabolic processes. The decrease in total liver weight suggests that this organ catabolizes its own tissue in order to supply the required structural material. The capacity of rat liver to respond most generously in this respect during periods of fasting has been demonstrated by Addis and coworkers (7, 8).

Our observation of elevated hepatic transaminase in pair-fed hypophysectomized rats treated with anterior pituitary growth hormone might be interpreted in several ways. A relatively low hepatic transaminase in the untreated hypophysectomized rat which has a high rate of amino acid catabolism (9) cautions us against the interpretation of increased oxidative deamination of amino acids by the two-step mechanism of Braunstein and Bychkov (2). Elevated amino acid concentrations in the skeletal muscle of pair-fed hypophysectomized rats in induced states of growth (1) and the parallel finding of elevated liver transaminase might be interpreted, however, as indicative of increased hepatic synthesis of amino acids from non-protein sources and subsequent transport of the amino acids to the muscle mass.

The finding of low skeletal muscle transaminase in induced states of growth and in rapidly growing normal animals (1) and the present observation of elevated transaminase in liver which is catabolizing its own tissue are in agreement with the view that transamination and protein synthesis are inversely related (10). Whether or not transamination controls protein synthesis by the removal of glutamic acid from the protein $\rightleftharpoons$ amino acid equilibrium (10) or by the rapid formation of glutamic acid (11) and other amino acids required for the synthesis of protein still remains to be determined.

SUMMARY

1. Treatment of normal adult female rats with anterior pituitary growth hormone produces no change in either hepatic or renal transaminase.

2. Hypophysectomy appears to have no effect on the hepatic or renal transaminase of the 26 to 28 day-old rapidly growing normal rat.

3. A fall in liver weight and an increase in hepatic transaminase were observed in pair-fed, hypophysectomized rats treated with anterior pituitary growth hormone and in normal rats maintained on a greatly restricted food intake by the paired feeding technique. Renal transaminase was unaffected.

4. Growth hormone had no effect *in vitro* on the transaminating capacity of either liver or kidney homogenates prepared from untreated hypophysectomized rats.

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DECARBOXYLATION OF L-GLUTAMIC ACID BY BRAIN*

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The metabolism of L-glutamic acid in brain has been of interest in so far as it is the only amino acid capable of maintaining respiration of brain (1, 2).

Recently Awapara *et al.* (3) and independently Roberts and Frankel (4) reported the presence of significant amounts of free γ -aminobutyric acid in brain of several animal species. This amino acid is known to be formed in bacteria (5) and in some plants (6) by the decarboxylation of glutamic acid. This reaction, however, has never been reported to occur in tissues of higher forms of life. The decarboxylation of cysteic acid (7) and dopa (8) has now been proved with certainty. Preliminary experiments (3) indicated that γ -aminobutyric acid was formed on incubation of glutamic acid with rat brain homogenates. The results reported here show that a decarboxylation occurs as indicated by the production of carbon dioxide under anaerobic conditions and the parallel increase in γ -aminobutyric acid as shown by means of filter paper chromatography.

EXPERIMENTAL

The work reported here was all done with crude homogenates of rat brain. The animals were decapitated and the brain removed and homogenized with sufficient ice-cold water to make a 20 to 30 per cent homogenate. For the manometric experiments, the system employed was as follows: homogenate plus water or inhibitor to make 1 ml. volume and 1 ml. of phosphate buffer, 0.25 M, in the main chamber of a Warburg flask with a single side arm. The center well contained 0.3 ml. of N sulfuric acid, and the side arm contained either glutamic acid solution neutralized with sodium hydroxide or water. The experiments were run at a temperature of 37.8° and the manometers were gassed with nitrogen. After equilibration the contents of the side arms were added to the main chamber and, at the end of the desired time, usually 90 minutes, the sulfuric acid was poured from the center well into the main chamber and after equilibration the final amount of CO₂ evolved was calculated. For the chromatographic determinations of γ -aminobutyric acid in connection with pH optimum determination, systems similar to those used in the manometric experiments (except that sulfuric acid was omitted) were set

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up in Thunberg tubes with the homogenate plus buffer in the tube and the substrate or water in the stopper. These tubes were filled with nitrogen by repeated evacuation and refilling, and were incubated and tipped along with the corresponding manometric run. At the end of the run the contents of the Thunberg tubes were used for pH determinations and then precipitated with alcohol and processed for the determination of γ -aminobutyric acid by the usual method (9).

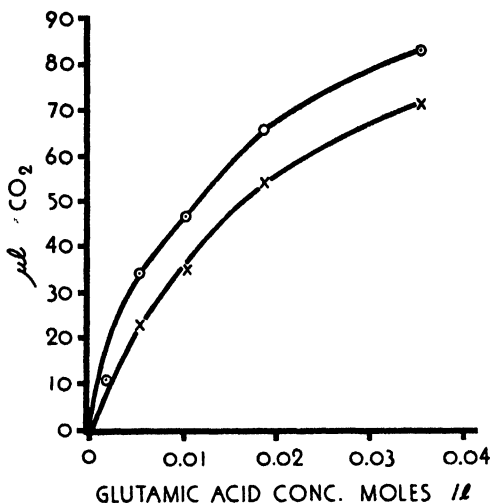


FIG. 1. Variation of reaction rate with concentration of glutamic acid. Each manometer vessel contained 1 ml. of brain homogenate (3620 mg. of brain + 10 ml. of H_2O), 1 ml. of 0.25 M phosphate buffer, pH 6.62. Incubation, 90 minutes. The side arms contained 0.4 ml. of glutamate (water in the case of the blank). ○, plotted on the assumption that all CO_2 from the blank is derived from decarboxylation of glutamic acid; ×, on the assumption that none of CO_2 from the blank is derived from glutamic acid. Concentrations of glutamic acid are corrected for glutamic acid content of homogenate.

RESULTS AND DISCUSSION

After having established that decarboxylation does occur when 0.01 M glutamic acid is used, the dependence of the reaction upon glutamate concentration was determined. The results are shown in Fig. 1. The Michaelis constant for the enzyme in this system is approximately 0.021 M. In subsequent experiments, 0.2 M glutamate solutions were used in the side arm. The linearity of the reaction with time was studied and was found to be linear for 2 hours, but not for 3. The results of two typical runs are shown in Fig. 2. The rate of reaction was found to be dependent upon enzyme concentration, as shown in Fig. 3. However, the curve does not intersect the origin, suggesting a dilution effect.

The optimum pH value was determined by two different methods; namely, manometrically and by paper chromatography. A set of blanks without glutamic acid was run for the determination of γ -aminobutyric acid together with a set of samples containing the same amount of glutamic acid as the manometric runs. The difference between these two curves should correspond to the curve obtained by the manometric method. Fig. 4 shows that this is approximately true. The optimum pH is about 6.8.

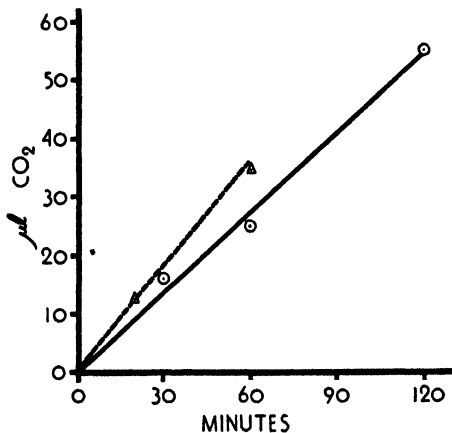


FIG. 2

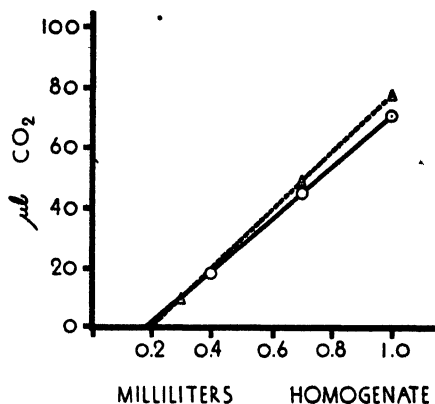


FIG. 3

FIG. 2. Linearity with time of decarboxylation of glutamic acid by brain homogenates. O, 1 ml. of homogenate (1940 mg. of rat brain + 10 ml. of H₂O); Δ, 1 ml. of homogenate (1900 mg. of rat brain + 9 ml. of H₂O); 1 ml. of 0.25 M phosphate buffer, pH 6.71. The side arms contained 0.4 ml. of 0.2 M glutamate for samples, 0.4 ml. of H₂O for blanks.

FIG. 3. Effect of volume of homogenate used on extent of decarboxylation of glutamic acid by brain. O, homogenate, 1850 mg. of rat brain + 5 ml. of H₂O; Δ, homogenate, 1830 mg. of rat brain + 4 ml. of H₂O; 1 ml. of 0.25 M phosphate buffer, pH 6.71. The side arms contained 0.4 ml. of 0.2 M glutamate for samples, 0.4 ml. of H₂O for blanks. Incubation, 90 minutes.

Finally, the action of some inhibitors was investigated, *viz.* cyanide, hydroxylamine, semicarbazide, and octyl alcohol; also, aspartic acid was investigated as a competitive inhibitor. In these tests the inhibitor was placed in the main chamber, replacing a portion of the homogenate. In the uninhibited runs accompanying these tests, water was used instead of the inhibitor. (The results obtained are shown in Table I.) Cyanide and the ketone reagents had an inhibiting effect, whereas octyl alcohol did not significantly influence the reaction. In this respect the enzyme resembles the other decarboxylases of mammalian tissue (10). Aspartic

acid exerts a significant inhibition but is not attacked by the enzyme, as demonstrated in separate experiments.

The occurrence of glutamic acid decarboxylase in brain, with the resulting formation of γ -aminobutyric acid from glutamic acid, appears to

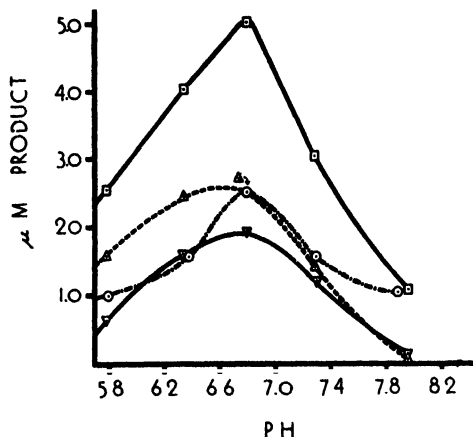


FIG. 4. Effect of pH upon decarboxylation of glutamate by brain homogenate. Each vessel or Thunberg tube contained 1 ml. of homogenate (3680 mg. of rat brain + 20 ml. of H_2O) and 1 ml. of 0.25 M phosphate buffer. The pH was determined electrometrically in Thunberg tube reaction mixtures at the end of the experiment. The side arms contained 0.4 ml. of 0.2 M glutamate for samples, 0.4 ml. of H_2O for blanks. □, γ -aminobutyric acid in the samples with added glutamic acid; ○, γ -aminobutyric acid in the blanks; Δ, net γ -aminobutyric acid; and ∇, manometric CO_2 .

TABLE I
Effect of Inhibitors on Decarboxylation of Glutamic Acid by Brain

Inhibitor	Final concentration	Inhibition
	M	per cent
HCN.....	10^{-4}	48
Hydroxylamine.....	10^{-4}	52
Semicarbazide.....	10^{-4}	23
Octyl alcohol.....	0.01 ml. per flask	4
Aspartic acid.....	5.7×10^{-3}	22
“ “.....	1.4×10^{-3}	30
“ “.....	2.9×10^{-3}	56

explain the presence of free γ -aminobutyric acid in brain. This reaction may offer a partial explanation for the fact that glutamic acid fails to accumulate in brain after intravenous administration (11). In most cases total amino acid nitrogen was measured by the method of Van Slyke

which is specific for α -amino acids, thus failing to measure the γ -aminobutyric acid which could be formed immediately after glutamic acid has entered the brain.

SUMMARY

The enzyme glutamic acid decarboxylase can be demonstrated in brain. In rat brain it has a pH optimum of 6.8 and a Michaelis constant of 0.021 M. It is active in homogenates for 2 hours and is inhibited by cyanide, semicarbazide, and hydroxylamine, but not by octyl alcohol. It is not active toward aspartic acid but is inhibited by aspartic acid in fairly large concentrations.

The authors wish to acknowledge the assistance of Mr. Robert Fuerst and Miss Nancy Lee Anderson in the performance of this work.

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PYRUVATE METABOLISM OF NORMAL RABBIT BONE MARROW IN VITRO

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The importance of pyruvic acid as an intermediary in carbohydrate metabolism has been well established and excellent studies have already been made of the pyruvate utilization of a number of tissues *in vitro* (1-4). Recently it has been reported by others (5) that pyruvate is rapidly utilized by bone marrow, but in view of the importance of marrow as the principal blood-forming tissue, it was considered of interest to reexamine and extend the study. In particular, it was hoped that the following questions might be answered: How much is the respiratory quotient affected by the addition of pyruvate to marrow cells? What is the metabolic fate of the pyruvate disappearing under both aerobic and anaerobic conditions?

EXPERIMENTAL

Methods

The methods employed in this study were essentially the same as we have described previously (6). Manometric data were obtained with the Summerson constant volume differential manometer (7). Bone marrows were taken from normal rabbits and were prepared as cellular suspensions in Ringer's solution. To each manometer vessel were added 1.0 ml. of marrow suspension and exactly 1.0 ml. of Ringer-bicarbonate solution. The manometer vessels were gassed for 20 minutes with a 95 per cent oxygen and 5 per cent carbon dioxide gas mixture in the aerobic experiments, and with a 95 per cent nitrogen and 5 per cent carbon dioxide mixture in the anaerobic experiments. The pH of the vessel contents was between 7.1 and 7.4. In these experiments 1.0 ml. of freshly distilled commercial pyruvic acid was diluted to 100 ml. in a volumetric flask and then neutralized with 50 per cent sodium hydroxide solution to pH 7.4 as measured with a Beckman pH meter. Of this, 0.2 ml. was tipped into the bone marrow suspension at the beginning of the experimental

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period. The vessels were shaken at a rate of 120 oscillations per minute in a water bath at 37.5°. The experimental period was 2 hours.

At the end of the experimental period the marrow was killed with 0.2 ml. of 2.5 *N* hydrochloric acid. The contents of each control vessel and of each experimental vessel were transferred quantitatively to a 25 ml. volumetric flask and the marrow protein was precipitated with trichloroacetic acid. The protein-free filtrates of both control and experimental vessels were analyzed for pyruvic acid by the method of Friedemann and Haugen (8), and for lactic acid by the method of Barker and Summerson (9). Utilization of pyruvic acid was considered to have occurred when the analyses indicated a greater quantity of pyruvate in the control vessel. Production of lactic acid was regarded as having taken place when the analyses showed a greater quantity of lactic acid in the experimental than in the control vessels. A Klett-Summerson photoelectric colorimeter was used in these determinations. In fourteen aerobic experiments, pyruvate utilization was measured by the manometric method of Westerkamp (10). The enzyme decarboxylase used in these experiments was obtained from brewers' yeast.

In this paper, oxygen consumption is given in c.mm. per mg. of cell protein per hour. Total acid production is expressed in c.mm. of carbon dioxide liberated from bicarbonate per mg. of cell protein per hour. Pyruvate utilization and lactate production are given in micrograms per mg. of cell protein per hour. In all cases the quantity of cell protein has been calculated by multiplying the total cell nitrogen, as obtained by micro-Kjeldahl analysis, by the factor 6.25.

Results

The histogram of Fig. 1 summarizes graphically the results of thirty-six analyses for aerobic pyruvate utilization. The average utilization was 5.7 γ of pyruvate per mg. of cell protein per hour. Twenty-two analyses were made by the method of Friedemann and Haugen, and fourteen by the method of Westerkamp. The recovery error from each of these methods averaged 4 per cent.

The results of the analyses for lactic acid are given in Fig. 2. The average amount of lactate formed aerobically in forty-one experiments was 3.1 γ per mg. of cell protein per hour, with an average recovery error of 4 per cent. We have shown (11) in a previous study that, in the absence of added glucose, marrow actually utilizes small amounts of endogenous lactic acid. It is clear, therefore, that the lactate formed in these experiments comes from pyruvate. The mean value of lactate formed represents 55 per cent of the average pyruvate consumed. The remain-

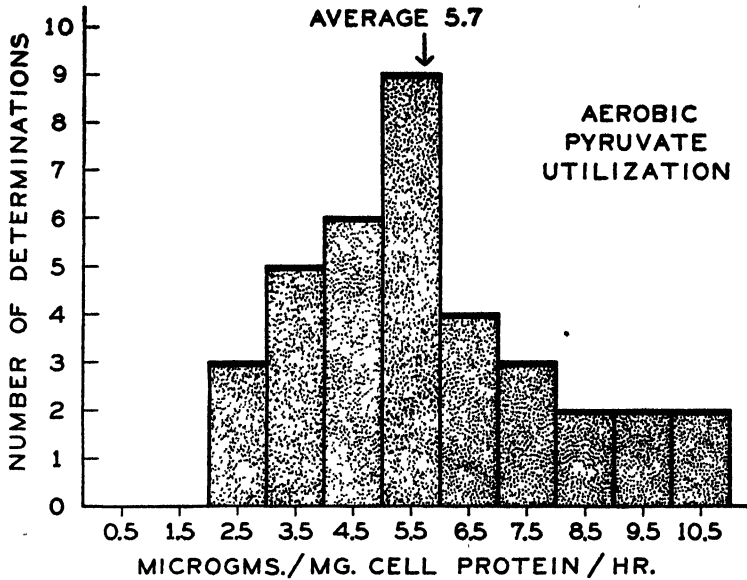


FIG. 1. The distribution and mean of thirty-six analyses for aerobic pyruvate utilization.

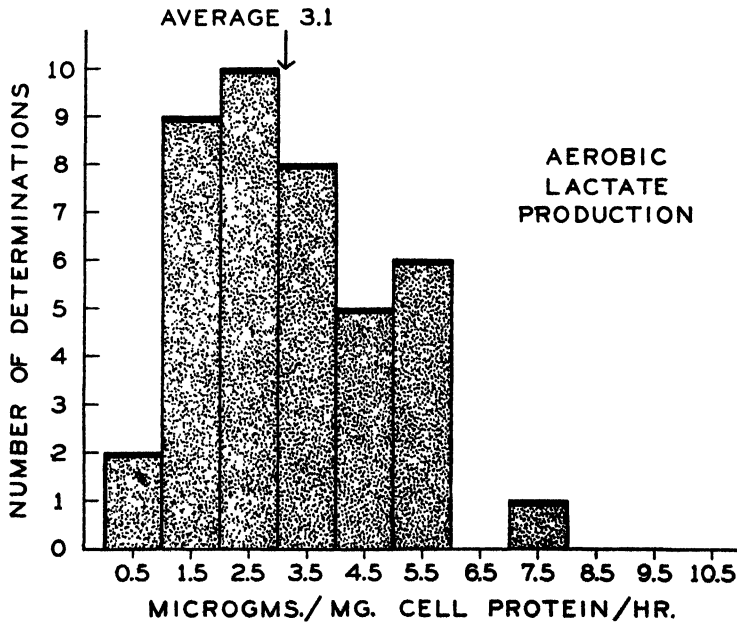


FIG. 2. The distribution and mean of forty-one analyses for aerobic lactate production.

ing or residual pyruvate is presumably utilized for purposes other than lactic acid formation.

Fig. 3 shows a comparison of the respiratory quotients of rabbit marrow in the presence and in the absence of pyruvate. The upper histogram presents the average and distribution of the respiratory quotients of 76 determinations in which neither pyruvate nor glucose was added to the marrow. In these experiments, which we have previously reported

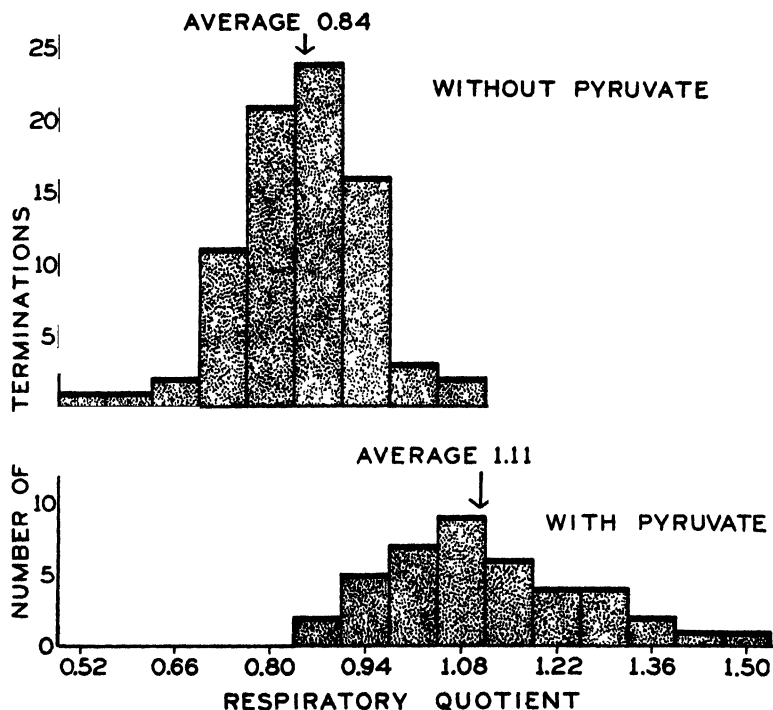


FIG. 3. Comparison of the mean and distribution of the respiratory quotients of rabbit marrow in the absence and presence of added pyruvate.

(11), the respiratory quotient averaged 0.84. The lower histogram shows the results of forty-one aerobic determinations obtained in the present investigation. As may be seen, the addition of pyruvate raises the average R. Q. to 1.11. Moreover, the distribution is shifted so that every R. Q. is higher than the average obtained in the absence of pyruvate. The elevation of the respiratory quotient indicates clearly that the metabolism of marrow cells is affected markedly by the addition of pyruvate.

In the forty-one aerobic experiments reported here, the average oxygen consumption was 2.9 c.mm. per mg. of cell protein per hour. This con-

sumption was lower by 0.6 c.mm. than the average we have previously reported (11) for rabbit marrow to which neither glucose nor pyruvate was added. Statistical analysis suggests that this decrease in oxygen consumption is significant. In these experiments, acid production liberated 0.2 c.mm. of carbon dioxide from bicarbonate per mg. of cell protein per hour. In the presence of neutralized sodium pyruvate, utilization of each molecule of pyruvate is accompanied by formation of a molecule of base. The over-all liberation of carbon dioxide from bicarbonate is therefore interpreted to mean acid formation in excess of pyruvate utilized.

The results of the anaerobic experiments with and without pyruvate are summarized in Table I. The average values are given, and along with them, for whatever they are worth, are added the statistical corrections. The significance of the standard error of the mean¹ is that the chances are 2:1 that the true mean lies within the limits specified. As

TABLE I
Anaerobic Metabolism of Normal Rabbit Bone Marrow in Ringer-Bicarbonate Medium at pH 7.3

	With pyruvate		Without pyruvate	
	Average $\pm \sigma$ mean	No. of deter- mina- tions	Average $\pm \sigma$ mean	No. of deter- mina- tions
Total acid production, c mm. per mg. per hr.	1.5 $\pm$ 0.15	20	1.0 $\pm$ 0.15	13
Pyruvic acid utilization, γ per mg. per hr.	4.2 $\pm$ 0.55	19		
Lactic acid production, γ per mg. per hr.	5.7 $\pm$ 0.54	20	1.0 $\pm$ 0.08	26

may be seen in Table I, the total anaerobic acid production was essentially the same in the presence or absence of pyruvate. The acid produced anaerobically in the presence of neutralized sodium pyruvate is interpreted as representing acid formed in excess of pyruvate utilized; the acid produced anaerobically in the absence of pyruvate is interpreted as being due largely to the formation of acids other than lactic acid. When pyruvate was added to the marrow, utilization averaged 4.2 γ per mg. of cell protein per hour, whereas lactate production averaged 5.7 γ per mg. of cell protein per hour. Recovery errors averaged 3 and 4 per cent respectively. Experiments performed under identical anaerobic conditions but without added pyruvate show that marrow can form lactic acid from endogenous materials. If lactate production from intracellular sub-

¹ σ of the mean = $\sqrt{\Sigma(v - M_v)^2/n(n-1)}$ where $\Sigma(v - M_v)^2$ is the sum of the squares of the individual deviations from the mean, and n is the number of analyses.

stances continues at the same rate after the addition of pyruvate, the lactate attributable solely to pyruvate may be considered to be 5.7 — 1.0 or 4.7 γ of lactate. The proximity of this figure to the 4.2 γ of pyruvate utilized suggests that an anaerobic pyruvate-lactate ratio approximating 1:1 exists in marrow.

DISCUSSION

Soskin and Levine (12) have criticized the concept of the respiratory quotient as giving an unreliable indication of metabolic events occurring either *in vivo* or *in vitro*. On the other hand, there is a large body of evidence relating the R. Q. to the utilization of particular food substances. Complete oxidation of pyruvic acid should produce an R. Q. of 1.2, and indeed it has been shown by others that many tissues *in vitro* exhibit R. Q. values that are near the theoretical value in the presence of pyruvate. Elliott and Schroeder (13) stated that pyruvate added to rabbit kidney cortex increased the R. Q. from 0.74 to 1.07. McGowan (2) reported an average R. Q. of 1.3 for pigeon brain mince suspended in Ringer-phosphate medium containing pyruvate. Gibson and Long (4) observed an average R. Q. of 1.2 in dialyzed ox heart suspensions when pyruvate was present.

The experimental results show clearly (1) that there is an increase in the respiratory quotient of marrow when pyruvate is added, and (2) that aerobic lactate formation comes wholly from pyruvate. A few experiments, not reported here in detail, show that lactate utilization by rabbit bone marrow is slow and is not associated with any large change in R. Q. The above facts together with the fact that oxygen consumption is not increased in these experiments suggest that pyruvate metabolism is substituted for, rather than superimposed upon, the metabolism of endogenous cellular materials. It may be inferred that only the residual pyruvate is concerned with the elevation of the respiratory quotient. This amount, which is the difference between the pyruvate used and the lactate formed, amounts to 2.6 γ of pyruvate. Complete combustion of this residual pyruvate is sufficient to account for 59 per cent of the oxygen consumed, and would theoretically elevate the R. Q. to 1.05. The approximate agreement of this value with the average experimental R. Q. of 1.11 is interpreted as supporting the concept of total combustion of the residual pyruvate. The immediate and rapid utilization of pyruvate by marrow suggests preferential carbohydrate oxidation; this appears to be accompanied by a simultaneous competitive inhibition of oxidation of other food substances. Krebs and Eggleston (1) have reported this effect in minced pigeon breast muscle. Summerson, Gilder, and Lee (14) did not find competitive inhibition in lymphosarcoma cells suspended in a Ringer-bicarbonate medium containing glucose. These investigators re-

ported that glucose was converted quantitatively to lactic acid, and that the R. Q. of 0.83 was associated with the combustion of endogenous cellular materials.

Anaerobically, bone marrow suspensions convert added pyruvate to lactate in a 1:1 ratio. Dismutation would require anaerobic carbon dioxide evolution and a pyruvate-lactate ratio of 2:1. There is no evidence in these experiments of any anaerobic carbon dioxide production that can be attributed to the addition of pyruvate, and it is therefore unlikely that dismutation is an important pathway in marrow, though this process has been shown to occur in testis and brain by Krebs and Johnson (15), and in rat brain by Weil-Malherbe (16). In the present investigation, no effort was made to determine acetic acid.

The experiments reported in this paper confirm the observations of Goldinger and coworkers (5, 17) that pyruvic acid is actively used by rabbit bone marrow cells *in vitro*. Comparison of their data with observations made in this study reveals important differences in the amounts of pyruvate and lactate used or produced. Furthermore, the pyruvate-lactate ratios of these authors are different from the ratios reported here.

Grateful acknowledgment is made to Dr. R. M. Bird for his friendly advice and help.

SUMMARY

1. Sixty-one experiments have been performed *in vitro* to determine the effect of pyruvate on the aerobic and anaerobic metabolism of rabbit bone marrow.

2. Aerobically, bone marrow converts approximately half of the pyruvate utilized to lactate. The other half is oxidized completely to carbon dioxide and water. Anaerobically, bone marrow cells convert all the pyruvate used to lactate.

3. In the presence of pyruvate the respiratory quotient of marrow averages 1.11. This contrasts with an R. Q. of 0.84 in the absence of pyruvate or glucose.

4. It is concluded from the aerobic experiments that (a) the R. Q. of marrow gives a reliable indication of the kind of material being metabolized by the marrow cells, and (b) pyruvate is preferentially metabolized by bone marrow.

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THE LIPIDE COMPOSITION OF THE LARGE GRANULES (MITOCHONDRIA) FROM RAT LIVER*

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The belief that mitochondria consist to a large extent of lipides, chiefly phospholipides, was stated by Mayer, Rathéry, and Schaeffer (1) on the basis of the behavior of mitochondria toward organic solvents and of the parallelism between the amounts of phospholipides chemically determined in the liver and the number and microscopic aspect of mitochondria under various experimental conditions. A similar parallelism has been noted since in other tissues (2, 3).

After the introduction of techniques for differential centrifugation in the separation of intracellular structures (4-7), chemical determinations showed that the "large granule" fraction has a high lipid content, and that much of its phosphorus is soluble in alcohol and ether. However, in a few instances in which the lipides were analyzed in some detail (8-10), relatively low values for typical phospholipides were found, suggesting the possibility of a peculiar composition of the lipides in the granules. Recently Hogeboom *et al.* (6) have elaborated what appears to be a definitive method for the isolation of mitochondria. Unlike previous methods, this technique yields preparations comparatively free from nuclei and cell fragments of larger size, as well as from submicroscopic particles. These preparations have been shown to carry on many of the essential oxidation processes of the cells (6, 7, 11, 12). Because of the interest aroused by the finding of a high lipid content in close association with important enzymatic systems, we thought it worth while to reinvestigate as thoroughly as possible the lipid composition of the "large granules," as prepared by a standardized technique.

EXPERIMENTAL

Preparation of Large Granule Fraction—Male albino rats, maintained on a stock diet (Rockland Farms) to 150 to 200 gm., were killed by de-

* With the technical assistance of M. L. Haigler. A preliminary report was presented before the Southeastern section of the Society for Experimental Biology and Medicine, Durham, North Carolina, January, 1950. This work was supported by the United States Atomic Energy Commission under a contract (No. AT-(40-1)-288, Title II) with the Bowman Gray School of Medicine of Wake Forest College.

capitation. The liver was rapidly removed, chilled, minced finely with scissors, and homogenized in 9 volumes of ice-cold 0.88 M sucrose solution. The granules were separated by differential centrifugation, according to the technique of Hogeboom *et al.* (6) as modified by Kennedy and Lehninger (7). After four washings with 0.15 M NaCl, the creamy tan precipitate was resuspended to a definite volume in NaCl solution. Aliquots for analysis were taken immediately, the total time elapsed from the death of the animals being 4 hours. The preparation consisted entirely of minute short rods and spherical granules, most of which stained with Janus green 1:10,000, which is the microscopic appearance described by Hogeboom *et al.* When tested under the conditions of Friedkin and Lehninger (13), the preparation was quite active in incorporating P^{32} into phospholipides.

Extraction of Lipides—Several procedures were tested comparatively on separate aliquots of the same suspension. When the suspension was extracted directly with boiling alcohol without previous precipitation with trichloroacetic acid (TCA), higher values for both the weight and the phosphorus of the lipides were obtained.¹ However, in the analyses reported here, precipitation and washing with aqueous TCA were included, since the elimination of the acid-soluble substances presumably favors the preparation of a purer lipid extract. The suspension of the granules was added to 2 volumes of ice-cold 10 per cent TCA. The precipitate was washed twice with 5 per cent TCA, dehydrated overnight in cold acetone, and then extracted for 5 to 6 hours with boiling alcohol in a semimicro apparatus of the Kumagawa-Suto type. The combined acetone and alcohol extracts were evaporated at reduced pressure and taken up in chloroform. The chloroform solution was filtered through asbestos and the filtrate brought to a definite volume. In order to estimate the proportion of unextracted lipides, the tissue residue after the acetone and alcohol extractions was saponified with 5 cc. of 45 per cent NaOH + 10 cc. of alcohol.

Chemical Determinations—For the *dry weight* determination, an aliquot of the suspension was dried to constant weight at 100° and the value corrected for the calculated amount of NaCl added with the suspension. Nitrogen (17) and phosphorus (18) were determined, after digestion, on the original suspension of granules (*total N and P*), on the combined TCA extract and washings (*acid-soluble N and P*), and on the chloroform extracts (*lipide N and P*). *Total proteins and nucleoproteins* were calculated ac-

¹ Similar observations have been previously reported in analyses of unfractionated liver (14, 15) and muscle (14), and recently also of the large granules of mouse liver (16). A detailed study of these discrepancies and of their possible interpretation is in progress in this laboratory.

cording to the formula, $6.25 ((\text{total N}) - (\text{acid-soluble N} + \text{lipide N}))$. The weight of the chloroform extract, dried at 55° , was indicated as *total lipides*. *Fatty acids and unsaponifiable matter* (by weight) and *total fatty acids* (by titration) were determined by a modified Kumagawa-Suto method (19), after saponification of the chloroform extract or of the extraction residue. In order to minimize the oxidation of highly unsaturated fatty acids, in the two experiments in which the *iodine number* of the fatty acids was determined (20), the unsaponifiable matter and the fatty acids were extracted separately with petroleum ether (21). *Total cholesterol* was determined after saponification of the lipide extracts, by the Liebermann-Burchard reaction in a chloroform medium (22). On another aliquot of the lipide extracts, the free cholesterol was first precipitated with digitonin in an acetone-alcohol-water medium (23). The cholesterol esters in the supernatant were saponified and the freed cholesterol determined as indicated (22). *Free cholesterol* was calculated by difference.

Choline was determined as the enneaiodide, after hydrolysis with HCl in methanol (24). The *various phospholipide fractions* were calculated from determinations of the total and alkali-labile P (25), total and alkali-labile choline (26), and ethanolamine and serine (27) in the chloroform extracts.

RESULTS AND DISCUSSION

The dry weights of the granules varied from 19 to 44 mg. per gm. of moist liver. These variations were apparently caused by variations in the centrifugal field, since the smaller yields were obtained when the larger volumes were handled and the speed of the centrifuge was somewhat lower (a Servall model SP centrifuge was the only refrigerated apparatus available). However, when the analytical data were expressed in per cent of the dry weight, the values, especially those for total P and N, were quite consistent (Table I), and also in good agreement with data in the recent literature (11, 12, 16). This suggests that the large granule fraction obtained in the various preparations by us, as well as by others who used the technique of Hogeboom *et al.*, has a well defined and approximately constant chemical constitution. This fraction would consist of 63 per cent proteins and nucleoproteins and 29 per cent lipides, the rest being represented by substances soluble in aqueous TCA. Our general procedure is essentially that proposed by Schneider (28) and used by several other investigators (7, 11, 25) for determining the phosphorus distribution in tissue, except that the extraction of the lipides should be much more effective. Even so, in most of our analyses comparatively large amounts of fatty acids were found by saponification of the extracted residues. It would appear that a notable portion of the lipides of

the mitochondria is present in unusually firm combinations with the proteins.

The average composition of the lipides extracted is given in Table II. Calculations of the lipid constituents from the proportion of their hydrolytic products present considerable uncertainties, since the same products often occur in more than one class of lipides. Subject to this reservation, it appears that phospholipides were the major components (79 per cent), the remainder probably being represented by neutral fat, cholesterol (one-half or more of which is free cholesterol), and other lipides. The high values for the iodine number of the fatty acids in the granules (values which are within the range of those reported for the phospholipide fatty

TABLE I
General Composition of "Large Granules" of Rat Liver

Values in per cent of the dry weight.

	Large granules						Unfractionated liver, averages*
	Preparation No.					Averages	
	3	4	5	6	9		
Nitrogen, total.	12.4	12.9	11.8	11.6	11.9	12.12	10.62
“ acid-soluble.	1.87	2.29	1.75	1.24	1.80	1.79	1.12
“ lipide	0.31	0.44	0.41	0.34	0.27	0.35	0.19
Phosphorus, total	1.66	1.36	1.36	1.31	1.19	1.38	
“ acid-soluble	0.11	0.19	0.19	0.21		0.18	
“ lipide	0.67	0.74	0.77	0.69	0.59	0.69	0.38
Proteins and nucleoproteins.	64.5	63.5	60.9	65.0	63.1	63.4	59.4
Lipides, extract (weight)	19.8	22.8	25.4	20.7	23.9	22.5	14.3
“ residue (fatty acids and unsaponifiable matter)	7.3	0.8	7.8	5.1	9.2	6.0	0.3

* These values are the averages of two or four determinations, made by the same procedures on the liver of rats placed under the same conditions as the rats from which the large granules were prepared.

acids of the unfractionated liver (29)) should be noted in view of the often postulated relationship between highly unsaturated fatty acids and specific functions of the liver cells. Among the individual phospholipides, our averages indicate that 45 per cent was lecithin, 8 per cent sphingomyelin, and 47 per cent "cephalin." In two analyses, most of the "cephalin" was phosphatidylethanolamine, little or no serine being present in the hydrolysate of the lipides. About one-third of the non-choline-containing phospholipides could not be identified as phosphatidylethanolamine, or as phosphatidylserine. This deficiency may be due to inadequacies in the analytical procedures, or perhaps to the presence of phospholipides with unidentified nitrogenous components (30, 31).

Our data do not offer any evidence for the presence in our lipid extracts of considerable amounts of lysophospholipides and cerebrosides, as suggested tentatively by Chargaff (10). The fatty acids determined are more than sufficient to account for the lipid P as typical phospholipides, and, in all but one of our analyses, the N:P ratio found corresponded very closely to that calculated from the proportion of sphingomyelin in the phospholipide mixture. In one of our preparations (No. 6), in

TABLE II

Per Cent Composition of Lipides Extracted from "Large Granules" of Rat Liver

	Large granules						Unfractionated liver, averages*
	Preparation No.					Averages	
	3	4	5	6	7		
Total fatty acids†... ..	66	64	60	69		64.7	66.2
Total fatty acids, iodine No.		119	126				
Non-phospholipide fatty acids‡... ..	11	13	13	15		13.0	21.2
Total cholesterol	4.68	4.85	4.42	3.29		4.4	4.6
Free "			2.28	2.56			
Total phospholipides§... ..	84.9	80.6	76.5	80.2	71.5	78.7	66.5
Choline-containing phospholipides .	44	60	56	56	47	53	57
Lecithin	40	50	40	51	43	45	50
Sphingomyelin	4	10	16	5	4	8	7
Non-choline-containing phospholipides	56	40	44	44	53	47	43
Phosphatidylethanolamine		25	29				23
Phosphatidylserine.. . . .		3	0				7
Ratio N:P. Calculated.. . . .	1.04	1.10	1.16	1.05	1.04	1.08	1.07
Found.....	1.03	1.34	1.18	1.10	1.03	1.14	1.15

* See explanation to Table I.

† Ml. of 0.01 N NaOH $\times$ 2.8 = mg. of fatty acids.

‡ Calculated by difference, (total fatty acids) - (phospholipide fatty acids).

§ Mg. of lipid P $\times$ 25 = mg. of phospholipides.

which the lipides were fractionated three times with acetone + $MgCl_2$, the precipitate contained about 95 per cent of the lipid P, with a molar ratio of fatty acids to P of 2.3. This value is much higher than one would expect if considerable amounts of either lysophospholipides or acetal phosphatides (9) were present. In addition, the lipid N of this extract was only slightly in excess of the value calculated for the N in the phospholipides, and no galactose was found to be present after hydrolysis of the lipides (32).

Since complete extraction of the large amounts of lipides in the granules

is apparently very difficult, the possibility of an incomplete extraction of cerebrosides, which are the least soluble of the lipides, cannot be excluded. However, it should be pointed out that the large granules, separated in saline medium by the older procedures, as in Chargaff's determinations, are contaminated with other cellular materials to a considerable degree (see also Huseby and Barnum (16)). It seems possible that cerebrosides (and perhaps also lyso- or acetal phospholipides) are concentrated in fractions other than the mitochondria.

Analyses of the unfractionated materials, from which the large granules were prepared, have not been made systematically. However, the data on the lipides of the mitochondria can be compared with the results of a few determinations which we have carried out with the same procedures on the whole liver of rats of the same strain and age and maintained on the same diet. The average results of these determinations are reported in the last column of Tables I and II. It appears that the mitochondria have a much higher content of lipides with a greater proportion of phospholipides, and, presumably, much less neutral fat. Except for these findings, the composition of the lipide mixture extracted from the large granules does not appear to be essentially different from that of the lipides of the whole tissue.

SUMMARY

Five preparations of large granules (mitochondria) separated from rat liver by differential centrifugation have been analyzed for their lipide content. Complete extraction of the lipides seems to be very difficult. Of the lipides extracted from the trichloroacetic precipitate, 79 per cent were phospholipides, 4.4 per cent cholesterol (one-half or more of which was free cholesterol), 17 per cent neutral fat and other lipides. The tentative distribution of the individual phospholipides was as follows: 45 per cent lecithin, 8 per cent sphingomyelin, and 47 per cent non-choline-containing phospholipides (most of which was phosphatidylethanolamine). Except for a higher content in lipides, especially phospholipides, the composition of the lipide mixture extracted from the mitochondria does not appear to be essentially different from that of the lipides of the whole unfractionated liver.

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INHIBITION OF SUCCINIC DEHYDROGENASE BY METHYLGLYOXAL*

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The reaction of the carbonyl group of methylglyoxal with —SH radicals of thiols has been the subject of numerous investigations (1–9). The reactivity of the keto aldehyde with —SH compounds suggested that a similar type of reaction may occur between methylglyoxal and —SH proteins. The physiological rôle of methylglyoxal as an inhibitor of —SH enzymes is of particular interest, since this keto aldehyde may be formed during carbohydrate metabolism (10–12). Mann and Quastel (13) have reported that other aldehydes have an inhibitory effect on brain metabolism. In this paper experiments are described which demonstrate that methylglyoxal can inhibit cell metabolism. The conditions of the inhibition and its possible significance in tissue metabolism are also discussed.

EXPERIMENTAL

It is well known that methylglyoxal is rapidly converted to lactic acid in crude tissue preparations (14, 15). Therefore, experimental conditions were sought under which the effect of methylglyoxal could be studied without the interference of the glyoxalase system. Schneider, Claude, and Hogeboom (16, 17) established that the succinate system of the rat liver is localized in the mitochondrial fraction. Since it was found in this laboratory that rat liver mitochondria in the presence of glutathione (GSH) do not convert methylglyoxal to lactic acid,¹ this enzyme preparation was chosen for the inhibition studies.

Materials—An aqueous solution of methylglyoxal was prepared from dihydroxyacetone² by the method of Neuberger *et al.* (18). The methylglyoxal concentration was determined according to Kuhn and Heckscher (19). The freshly prepared aqueous solution of methylglyoxal was neutral-

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¹ Unpublished experiments.

² Dihydroxyacetone, c.p., was obtained from the Delta Chemical Works, 23 West 60th Street, New York 23.

ized and diluted to appropriate concentrations immediately before being pipetted into the respirometer flasks. Rat liver mitochondria were prepared by a modified procedure of Schneider *et al.* (16, 17) as described by Kennedy and Lehninger (20). The freshly removed and chilled rat liver was ground up with 9 volumes of ice-cold 0.88 M sucrose. Large particles were removed by three successive centrifugations at $1500 \times g$ on the Servall angle centrifuge (type SS-1A), which was operated in the cold room at $0-4^{\circ}$. The mitochondria were agglutinated by the addition of 1.5 M KCl to give a final KCl concentration of 0.15 M. The sedimentation of mitochondria was carried out at $5000 \times g$ for 8 minutes, followed by three successive washings with 0.15 M KCl. During this procedure soluble —SH compounds were quantitatively removed, and all detectable (21) —SH radicals were found to be bound to the washed particles. The mitochondria were finally resuspended and homogenized in 0.15 M KCl to make 0.1 ml. of mitochondria suspension correspond to 100 to 120 mg. of total fresh liver weight.

Succinoxidase and cytochrome oxidase were tested manometrically, according to Schneider and Potter (22). Dehydrogenase activity was measured by the ferricyanide technique of Quastel and Wheatley (23).

Results

Site of Action of Methylglyoxal on Succinate System of Rat Liver Mitochondria—When a suspension of rat liver mitochondria was incubated with methylglyoxal, a progressively increasing inhibition of succinoxidase activity was observed. After a certain length of incubation complete loss of enzyme activity occurred. Results of such an experiment are shown in Table I. Succinate was added to the main compartment of the Warburg vessel containing the enzyme preparation simultaneously with methylglyoxal and after 15 or 30 minutes incubation of the mitochondria with the inhibitor.

When succinate and methylglyoxal were added simultaneously, the onset of enzyme inhibition was retarded. Similar protective effect of succinate was reported by Mann and Quastel (13), Hopkins and Morgan (24), von Euler and Hellström (25), and Potter and Dubois (26) for other inhibitors of succinic dehydrogenase which react with —SH radicals of this enzyme. In order to test the validity of the assumption that methylglyoxal combines with the —SH radicals of succinic dehydrogenase and thereby inhibits its catalytic activity, —SH analyses (21) were performed on the contents of respirometer flasks containing the same constituents, except for succinate, as in the succinoxidase experiment. As can be seen in Fig. 1, a rapid decrease of —SH content of mitochondria occurred when incubated with methylglyoxal.

TABLE I

Effect of Time of Incubation on Inhibition of Succinoxidase by Methylglyoxal

The results are expressed in microliters of oxygen in 15 minutes.

Time of manometer readings	Succinate and methylglyoxal added simultaneously		Succinate added after 15 min. incubation		Succinate added after 30 min. incubation	
	Experiment	Control	Experiment	Control	Experiment	Control
<i>min.</i>						
15	131	130				
30	69	128	20	110		
45	27	125	10	114	1	82
60	13	128	2	108	0	88

Each Warburg flask contained a suspension of rat liver mitochondria (corresponding to 100 mg. of fresh liver), cytochrome *c*, Ca^{++} , Al^{+++} , phosphate buffer, and succinate in the concentrations given by Schneider and Potter (22), and $46\ \mu\text{M}$ per ml. of methylglyoxal; 0.2 ml. of 20 per cent KOH in the center well, gas, air; temperature of bath 37° ; total volume of reactants, 3 ml.; time of equilibration 5 minutes.

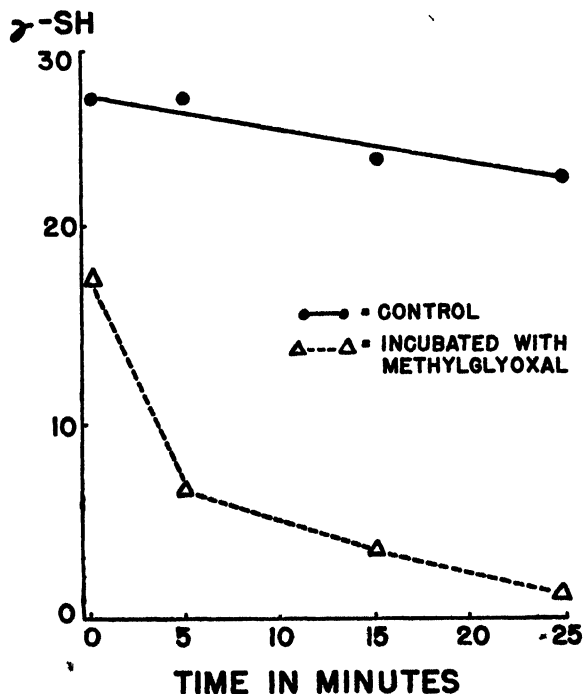


FIG. 1. Effect of incubation with methylglyoxal on the $-\text{SH}$ content of rat liver mitochondria. Each Warburg flask contained the same amount of enzyme, cytochrome *c*, Ca^{++} , Al^{+++} , phosphate, and methylglyoxal as described in the legend of Table I, but no succinate. At 0, 5, 10, 15, and 25 minute intervals one experimental flask (containing methylglyoxal) and control flask were instantly analyzed for $-\text{SH}$ groups, by carrying out the ferricyanide test of Anson (21) on 0.3 ml. samples.

Succinic dehydrogenase is the only known member of the succinoxidase system which requires —SH groups for its catalytic activity. The site of action of methylglyoxal on the succinate system was determined by comparing the rate of aerobic (22) and anaerobic (23) succinate oxidation in the presence of various concentrations of the inhibitor. When the percentage of inhibition of aerobic and anaerobic oxidation is plotted against the amount of inhibitor, the two series of experimental points (Fig. 2)

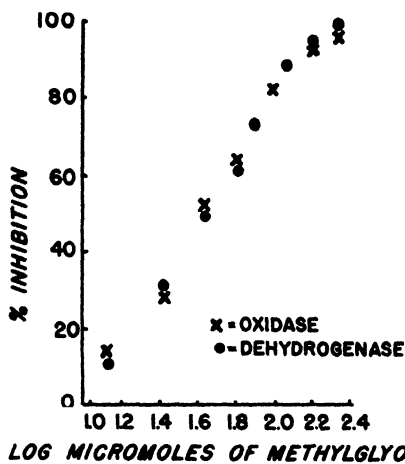


FIG. 2. Inhibition of succinic oxidase and dehydrogenase. The aerobic and anaerobic oxidation of succinate by rat liver mitochondria was measured after 20 minutes incubation with the inhibitor (methylglyoxal). At the end of incubation in the aerobic systems (22) succinate was added from the side arm, and succinate + ferricyanide (0.1 ml. of 0.5 M succinate + 0.1 ml. of 20 per cent potassium ferricyanide, dissolved in 1.3 per cent NaHCO_3) in the anaerobic systems (23). For each set of aerobic and anaerobic test systems blanks were set up containing no succinate but varying amounts of methylglyoxal. Corrections for such blank values were carried out routinely in all measurements reported in this paper. Each flask contained, besides constituents of the test system, a suspension of mitochondria, corresponding to 100 mg. of fresh liver. The rate of succinate oxidation was determined by averaging three successive 10 minute manometer readings which did not deviate from the mean more than ± 5 per cent. The reaction rates were linear in each case.

cannot be distinguished from each other, indicating that the percentage of inhibition is identical in both aerobic and anaerobic conditions. This observation strongly suggests that the anaerobic part of the succinate system is the limiting factor of the enzyme inhibition. This conclusion was also warranted by the finding that cytochrome oxidase (22) was unaffected by methylglyoxal in concentrations which inhibited the aerobic oxidation of succinate.

Conditions of Enzyme Inhibition—Since the inhibitory effect of methyl-

glyoxal was apparently due to its carbonyl group, it could be assumed that substances which react with this radical protect —SH enzymes against methylglyoxal inhibition. In agreement with this assumption it was found that, when rat liver mitochondria were incubated with methylglyoxal in the presence of semicarbazide, the inhibitory effect of methylglyoxal was greatly reduced. From a physiological view-point the most important compound which is known to react with methylglyoxal is GSH, the coenzyme of the glyoxalase system (2, 3, 14, 15, 27, 28). It is evident that GSH together with glyoxalase plays an important rôle in the

TABLE II
Effect of Semicarbazide, GSH, and Liver Extract on Inhibition of Succinic Dehydrogenase by Methylglyoxal

Experimental conditions	Per cent inhibition
Enzyme with and without semicarbazide HCl (0.01 M).	0
“ + methylglyoxal (46 μ M per ml.)	76–80
“ + semicarbazide HCl (0.01 M) + methylglyoxal	37
Enzyme + 1 mg. GSH + methylglyoxal	59
“ + 3 “ “ + “	21
“ + 5 “ “ + “	0
“ + 1 “ “ + 0.2 ml. liver extract + methylglyoxal	0
Enzyme + 0.2 ml. liver extract + methylglyoxal	52

Freshly prepared rat liver mitochondria (corresponding to 120 mg. of fresh liver) were incubated anaerobically for 20 minutes with and without the reactants listed in the table. Succinic dehydrogenase activity was measured by tipping in 0.1 ml. of 0.5 M Na succinate and 0.1 ml. of 20 per cent potassium ferricyanide from the side arms, and CO₂ formation was measured in 5 minute intervals for 30 minutes. Correction was made for ferricyanide reduction by GSH, semicarbazide, and liver extract. GSH alone had no effect on the freshly prepared enzyme. Final volume of reactants, 3 ml.; NaHCO₃ concentration 0.025 M; gas phase, 95 per cent N₂ + 5 per cent CO₂; temperature 37°.

protective system of tissues against methylglyoxal inhibition. Therefore the protective rôle of GSH was investigated separately and also in the presence of the glyoxalase enzyme system. It was found that the magnitude of inhibition of succinic dehydrogenase of mitochondria was a function of the GSH concentration in the absence of the glyoxalase enzyme system. The protective effect of GSH, similar to that of semicarbazide, did not change with time. The protective effect of GSH was markedly potentiated by liver extract. The liver extract was prepared by homogenizing 1 part of liver (by weight) with 2 parts of ice-cold 0.15 M KCl, then centrifuging for 30 minutes at 25,000 $\times g$ in the cold room. The supernatant had no

succinic dehydrogenase activity, but contained an active glyoxalase system. Calculated from the CO_2 evolved in the course of 20 minutes incubation, approximately one-half to two-thirds of the methylglyoxal was converted to lactic acid if GSH and liver extract were present simultaneously. The glyoxalase reaction came to an almost complete standstill after 20 minutes, a finding similar to that reported by Hopkins and Morgan (27). The partial protection by liver extract alone against methylglyoxal inhibition can be explained by the $-\text{SH}$ (GSH, $-\text{SH}$ protein, amino acid) content of the liver extract. Results of these experiments are summarized in

TABLE III
Effect of Malonate and Incubation with GSH on Reversibility of Inhibition of Succinic Dehydrogenase by Methylglyoxal

Experimental conditions	Before incubation with GSH		After 35 min. incubation with GSH	
	Enzyme activity*	Per cent inhibition	Enzyme activity*	Per cent inhibition
Control (no methylglyoxal, no malonate)	560	0	1230	0
Enzyme + methylglyoxal.....	38	95	100	92
“ + malonate + methylglyoxal..	264	53	830	32
“ + “	353	37	1200	0

Mitochondria (equivalent to 800 mg. of fresh liver) were incubated for 15 minutes with malonate (1.0 ml. of 0.5 M), methylglyoxal ($208 \mu\text{M}$), and malonate + methylglyoxal. Distilled water was substituted for malonate and methylglyoxal in the controls. Volume of reactants, 3 ml. After incubation mitochondria were sedimented at $9000 \times g$ and washed thrice with 5 ml. of 0.15 M KCl. The mitochondria were then resuspended in 3 ml. of 0.15 M KCl, and in 0.5 ml. samples enzyme activity was tested immediately and after 35 minutes incubation with 5 mg. of GSH under anaerobic conditions. The test system was the same as that described in the legend to Table II.

* Enzyme activity is expressed in terms of CO_2 evolved (corrected values) due to anaerobic succinate oxidation by a 0.5 ml. suspension of mitochondria (equivalent to 133 mg. of fresh liver).

Table II where the inhibitory effect of methylglyoxal is demonstrated on comparable amounts of enzyme under various experimental conditions.

In further experiments the reversibility of the inhibition of succinic dehydrogenase by methylglyoxal was investigated. The inhibitory effect of methylglyoxal was compared to the effect of malonate, which is a well known competitive inhibitor of succinic dehydrogenase (29). Equal amounts of rat liver mitochondria were incubated for 15 minutes in the presence of malonate, methylglyoxal, and malonate + methylglyoxal (methylglyoxal was added after malonate). The amount of malonate present (1 ml. of 0.5 M malonate in 3 ml. final volume of reactants) completely inhibited succinic dehydrogenase of the mitochondria. After 15 min-

utes incubation at 37°, the mitochondria were quantitatively sedimented by centrifugation at $9000 \times g$ for 20 minutes at 0°, repeatedly washed with portions of 0.15 M KCl, and then resuspended and rehomogenized in a final volume of 3 ml. Succinic dehydrogenase activity was determined by the ferricyanide technique (23) immediately after this procedure and also after anaerobic incubation of the mitochondria with 5 mg. of GSH. The results are shown in Table III. During the procedure of incubation and repeated washings without inhibitor, about 50 per cent of the succinic dehydrogenase was inactivated which could be completely reactivated by anaerobic incubation with GSH. The reversible inactivation of succinic dehydrogenase is readily explained by the reversible oxidation of —SH groups by molecular oxygen (30–32), which occurred in the course of handling the mitochondria. It is of interest that repeated washings and incubation with GSH did not reverse the inhibition of succinic dehydrogenase by methylglyoxal (95 per cent inhibition before, 92 per cent after incubation with GSH). Attempts to reverse the enzyme inhibition by incubation of the inhibited mitochondria with liver extract (0.2 ml. of 0.15 M KCl extract of rat liver) in addition to GSH were also unsuccessful. Malonate had a protective effect against methylglyoxal. The enzyme inhibition due to malonate alone could be completely reversed by repeated washings and anaerobic incubation with GSH.

DISCUSSION

Evidence presented in the experimental part of this paper strongly suggests that the inhibitory effect of methylglyoxal on succinic dehydrogenase is due to its reaction with the enzyme protein. It was found in preliminary experiments that other —SH enzymes (malic-, glutamic-, triosedehydrogenases, hexokinase, and adenosinetriphosphatase) present in rat tissue homogenates or extracts are also inhibited by methylglyoxal, similarly to succinic dehydrogenase.¹ The conditions of enzyme inhibition, as exemplified by the succinate system of rat liver mitochondria, are of particular interest. The GSH-glyoxalase system of the cell can completely protect —SH enzymes against the inhibitory effect of methylglyoxal. Once the reaction of the keto aldehyde with succinic dehydrogenase occurred, it was impossible to reverse the inhibition under our experimental conditions.

A tentative explanation of the apparently irreversible inhibitory effect of methylglyoxal can be offered. It is known that the rest of the thiol molecule plays an important rôle in determining the nature of the methylglyoxal-thiol complex. Schubert (8) has shown that, while GSH combines with methylglyoxal to form a labile addition compound, thiols like cysteine and thiourea, which have free amino radicals in the vicinity of their —SH groups, react with the carbonyl group of methylglyoxal with a loss of H_2O

to form a more stable condensation product. Such a condensation compound of cysteine and methylglyoxal has the property of changing its color to brown (8, 33) during incubation.

A darkening of the mitochondria could be observed upon incubation with methylglyoxal. It is, therefore, conceivable that methylglyoxal combines with $-SH$ enzymes to form a stable condensation compound, since the location of the enzyme $-SH$ group might be of the cysteine type. The stoichiometric relationship between methylglyoxal binding of enzyme $-SH$ groups and decrease of enzymatic activity must be determined with a crystalline $-SH$ enzyme. The protective effect of GSH in the absence of the glyoxalase enzyme system can readily be understood on the basis that GSH combines with the keto aldehyde to form a labile addition compound and thereby competitively inhibits the reaction of methylglyoxal with the $-SH$ groups of the enzyme protein.

It is evident that in the presence of GSH and glyoxalase methylglyoxal is rapidly detoxified. The first step of the enzymatic conversion of methylglyoxal to lactic acid is, according to Jowett and Quastel (3), the reversible combination of methylglyoxal and GSH. Yamazoye (9), and recently Racker (28), stated that in the presence of glyoxalase a different type of methylglyoxal-GSH complex is formed compared to the labile complex described by Schubert (8). Hopkins and Morgan (27) separated glyoxalase into two protein fractions. Racker (28) recently reported that the combination of GSH and methylglyoxal is catalyzed by a protein fraction of glyoxalase and the conversion to lactic acid is also brought about by a separate enzyme. It is possible that both enzyme fractions of the glyoxalase system participate in the inactivation of methylglyoxal, first by its combination with GSH, then its conversion to lactic acid.

The physiological rôle of glyoxalase in muscle tissue was suggested by Meyerhof (34), who pointed out that this enzyme system may eliminate the spontaneously formed methylglyoxal. The importance of this mechanism is underlined by the results described in this paper. Whenever the formation of methylglyoxal is increased, or its detoxification impaired, the inactivation of $-SH$ enzymes by methylglyoxal may occur. Further studies are now being carried out to determine the occurrence and rôle of methylglyoxal in abnormal cell metabolism.

SUMMARY

1. Upon incubation with methylglyoxal an inhibition of the aerobic and anaerobic oxidation of succinate by rat liver mitochondria occurs. The inhibition of succinic dehydrogenase by methylglyoxal was shown to coincide with the disappearance of $-SH$ groups of the mitochondrion particles.

2. Succinate, malonate, semicarbazide, and GSH have a protective

effect against the inhibitory effect of methylglyoxal. Semicarbazide and GSH protect the enzyme by complex formation with methylglyoxal.

3. Rat liver extract, containing the glyoxalase enzyme system, potentiates the protective effect of GSH.

4. The inhibition of succinic dehydrogenase by methylglyoxal could not be reversed by repeated washings and anaerobic incubation of the inhibited enzyme with GSH, even in the presence of glyoxalase.

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ULTRAVIOLET ABSORPTION SPECTRA AT REDUCED TEMPERATURES

I. PRINCIPLES AND METHODS*

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Low temperature absorption and fluorescence spectra of solids, liquids, and solutions often reveal increased spectral detail of use in analytical procedures and molecular structure studies. Nevertheless, while qualitative observations of the influence of liquid air temperatures upon optical properties were undertaken very early (1, 2), investigations of the absorption and fluorescence of organic compounds at the temperature of liquid nitrogen (-195.6° ; 77.4°K.) and below have appeared only sporadically (3-25). Because of the potential usefulness of the technique we have undertaken a systematic study of the low temperature spectra of substances of biochemical interest. The present paper discusses the methods employed; subsequent papers will deal with the experimental results. In this work, we have emphasized the wave-length location of absorption bands and the accurate determination of relative optical densities rather than precision in the determination of absolute optical densities, thus permitting the use of simpler methods than would otherwise be necessary.¹

Principles of Method

Whereas molecules in the vapor state yield complex band spectra (26), the spectra of molecules in the condensed state usually lack any appreciable detail attributable to individual vibrational or rotational energy

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¹ It should be noted that infra-red spectroscopy is usually accomplished without determining absolute densities; somewhat analogously, emission spectroscopy usually involves the absolute determination of line wave-lengths with only relative estimates of line intensities. Absolute density measurements are of the greatest use in the quantitative determination of previously identified and characterized pure substances or mixtures thereof, whereas the positions of absorption bands and their relative density values are of much greater use for the identification of unknown substances and molecular structure studies.

changes; thus the lines of the benzene vapor spectrum are largely obliterated in its solution spectrum ((27) chapter 8). Among the factors that contribute to lack of detail in spectra are the following (26, 28-30): (a) the Doppler effect, (b) collision damping, (c) multiplicity of permitted energy transitions, (d) random variation of internal Stark and Zeeman effects, (e) predissociation or dissociation, and (f) natural line breadths. The effects of all but the last, which is relatively unimportant, are decreased by lowering the temperature of the absorbing substance.

The statistical randomness of the internal Stark and Zeeman effects may be attributed to two causes: (a) random distribution of absorbing groups, polar groups, ions, etc., in space at any particular instant, and (b) thermal motion of these entities during the period of observation. While the former effect may be decreased by examining substances in well ordered states (*e.g.* crystals), it may in most instances be expected to cause some diffuseness of the condensed state spectra even at the lowest temperatures. Another factor that may limit the appearance of greater spectral detail upon refrigeration is internal damping (8-11). If the structure of the molecule is such that the absorbing chromophore may transfer energy to other parts of the molecule, the life time of the excited states may be reduced and the absorption bands broadened.² In favorable instances, these perturbing effects are insufficient to prevent a marked increase in spectral detail of biochemical substances at reduced temperatures, as will be apparent from the experimental results to be reported.

Preparation of Specimens

The preparation of suitable samples is a major technical problem in low temperature absorption spectroscopy and considerable effort has therefore been devoted to investigating old and new specimen preparation methods.

Ultraviolet absorption spectra of biochemical compounds are usually obtained with solutions in transparent liquid solvents such as water. At low temperatures, freezing, crystallization, and cracking of solvents often lead to the formation of semiopaque masses of high light-scattering power. For satisfactory results, the solvent, solute, cell design, and condition of refrigeration must be such that the cooled solution vitrifies to yield a reasonably transparent glass containing the solute in molecular dispersion.³ Films of water solutions less than about 0.05 mm. in thickness

² It may be shown by Fourier analysis (31) that shortening or damping of an oscillatory wave-train results in the broadening of its corresponding frequency spectrum; only an infinite wave-train of constant amplitude corresponds to a single frequency. Thus damping of molecular oscillations as a result of collisions (collision damping) or energy transfer would be expected to broaden the corresponding spectrum line.

³ An alternative is to use solvents that remain liquid at 77 °K., such as equal

form vitrified solids when cooled rapidly by immersion in liquid nitrogen, but these have limited use. Special solvent mixtures are better adapted for most low temperature investigations.

A mixture of ether, isopentane, and alcohol (EPA) developed by Lewis and coworkers (9) for spectroscopy in the visible region was found usable in the ultraviolet if the solvents were purified with sufficient care.⁴ This mixture has satisfactory optical properties at 77 °K. in the proportions Lewis recommended: 5 parts of ether, 5 parts of isopentane, and 2 parts of alcohol. As Lewis suggested, the proportions may be varied somewhat to suit the solubility characteristics of different compounds or for work at different temperatures. To facilitate solution, the solute is first dissolved in the alcohol, and the ether and isopentane are then added. At 77 °K., EPA contracts by about one-third its original volume. In cells of 1 cm.

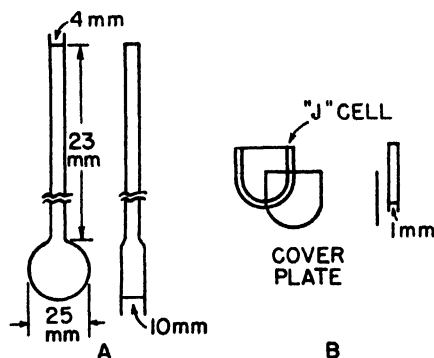


FIG. 1. A. Quartz absorption cell used for low temperature spectra of solutions in ether, isopentane, and alcohol (EPA). B, modified type J Hilger quartz absorption cell used for low temperature spectra of solutions in glycerol and water.

thickness or less, the vitrifying solution coheres and contracts toward the side walls, leaving one deep cleft down the center with sufficient clear area at one side for transmission of the light beam. The special quartz cell⁵ that we have used for EPA is shown in Fig. 1, A. The long stem prevents liquid nitrogen from entering when the cell is immersed, and the

parts of propene and propane alone or mixed with 1:10 of *n*-propyl alcohol, as used by Freed *et al.* (25,32).

⁴ Isopentane is purified by passage through a column of activated silica gel. Alcohol and ether are purified by the method of Castille and Henri (33), an eleven plate Brunn column being used for the final fractional distillation. When the solvents are thus purified, the mixture can be used to 2200 Å with no interfering absorption bands, and with only a gradual rise in absorption toward short wave-lengths, amounting to an optical density of 0.2 at 2200 Å for a 1 cm. path.

⁵ Constructed by the Nieder Fused Quartz Works, Wellesley, Massachusetts.

open construction minimizes breakage as a result of expansion and contraction.

A mixture of 1 part of methycyclohexane to 3 of isopentane, also used by Lewis (9), is satisfactory for biochemical substances of suitable solubility. Low temperature spectra of compounds in this solvent and in EPA showed no differences attributable to the solvents.

The 3:1 glycerol-water mixture used by Broda and Goodeve (34) was found to be satisfactory for many polar substances insoluble in EPA. This mixture has been used by Haas (22) for low temperature studies of proteins and amino acids, to be described in a later communication. Because of excessive cracking, the mixture is unsatisfactory at 77 °K. in the 5 cm. path length used by Broda and Goodeve. If the path is restricted to about 1 mm. (as in the modified type J Hilger cell⁶ shown

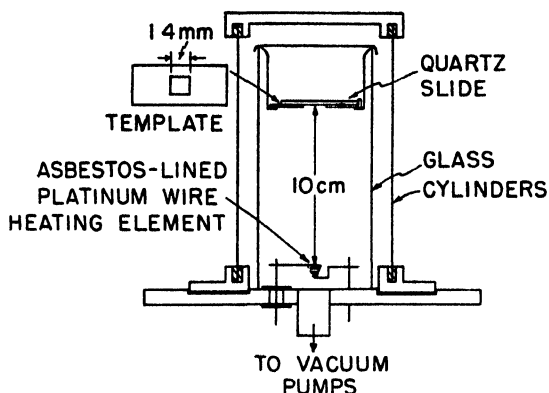


Fig. 2. Vacuum apparatus for preparing evaporated solid films of organic substances for low temperature spectroscopy.

in Fig. 1, B), cracks form perpendicular to the cell windows, leaving sufficient clear area for satisfactory use.

At liquid hydrogen temperature (-252.7° ; 20.5° K.), use of vitrifying solvents is considerably more difficult and no suitable mixtures have yet been devised for use at liquid helium temperature (-268.9° ; 4.3° K.). Furthermore, polar constituents in solvent mixtures (essential when highly polar biochemical substances are to be dissolved) may result in undesirable masking of spectrum detail. To overcome these difficulties, thin solid films of pure substances may be used.

Homogeneous films of substances of sufficiently low molecular weight may be prepared by molecular sublimation onto quartz slides in a vacuum

⁶ Obtained from the American Instrument Company, Silver Spring, Maryland.

chamber (about 10^{-3} mm. pressure) of the type used for shadow-casting electron microscope specimens. The material to be sublimed is placed in a small asbestos cup (capacity about 20 mg. of powdered material) inside a conical helix of platinum wire suspended between terminal posts (Fig. 2). The lining is built up by filling the helix with an aqueous asbestos suspension and gradually vaporizing the water by passage of current through the helix. The quartz slide is held in a copper template that masks all but a square central area, 1.4 ± 0.04 cm. on a side, and is suspended directly above the evaporating cup at a distance of about 10 cm. To reduce the possibility of thermal decomposition, sublimations are carried out at the lowest temperature (lowest filament current) practicable. For the polar substances of high melting point to which this technique has been primarily applied, currents of 1 to 2 amperes are necessary, corresponding to power inputs of 0.1 to 0.5 watt. As the current is increased cautiously, small particles begin to stand on edge or to bounce about in the molecular stream; additional evidence of sublimation is soon obtained from deposits on the chamber walls and the slide.

At a thickness corresponding to an optical path length of $\lambda/4$ for visible light, the sample begins to exhibit interference colors. With increasing thickness, the regular sequence of Newton's colors is seen, passing eventually into higher orders, and the deposition of material on the slide can be followed quantitatively by observing these colors. After a few films had been made, it proved possible to estimate accurately the interference color corresponding to a thickness that would yield a usable optical density (0.5 to 1.5) for a film of a new material from knowledge of its absorption coefficient in solution.

If absolute solid state absorption coefficients, α , are to be determined with films, it is essential to know the film thickness, x , for substitution in the Bouguer-Lambert law expression, $\alpha x = \log_{10} (I_0/I_s)$, where $\log_{10} (I_0/I_s)$ is the optical density.⁷ For comparison with solution spectra, however, it is more useful to know the weight per sq. cm., δ ; this corresponds to the product αx in the Bouguer-Lambert-Beer equation, $Kc x =$

⁷ The film thickness may be determined precisely from interference colors if the refractive index is known. Measurement of the index is possible but may be inconvenient unless appropriate facilities are available. If a reasonable value of index such as 1.5 is assumed, a close approximation to the film thickness may be obtained. Thus, for thymine, a film with an optical density of 0.9 at 2800 Å showed a first order interference color of light yellow, indicating an optical thickness of about 1200 Å and therefore a physical thickness of about 800 Å. Suitable films of other pyrimidines and of purines are of similar thickness. Films of tyrosine, which has a considerably lower absorption coefficient, are necessarily thicker; a tyrosine film of optical density 0.8 showed a third order interference color of red, indicating an optical path length of about 7000 Å and a physical thickness of about 4700 Å.

$\log_{10} (I_0/I_x)$, where K is the absorption coefficient, c the concentration (gm., moles, etc., per ml.), and x the path length (cm.). The value of δ may be determined easily by washing off a known area of film into a known volume of suitably buffered solvent and measuring the optical density by usual methods. Thus a film of cytosine of area 2.023 sq. cm. had a density of 0.595 at 2670 Å (Beckman determination) in 1 cm. path length when dissolved in 4 ml. of water buffered at pH 6.5. A separate solution of cytosine made up in the same buffer at a concentration of 15.8×10^{-6} gm. per ml. had a density of 0.895 at 2670 Å. Hence the weight per sq. cm. of the original film was $(4/2.023) \times 15.8 \times 10^{-6} \times (0.595/0.895) = 20.7 \times 10^{-6}$ gm. per sq. cm. The film itself had an optical density of 0.900 at 2580 Å, and since $\delta = 20.7 \times 10^{-6}$, the film absorption coefficient was 0.900 per $20.7 \times 10^{-6} = 4.35 \times 10^4$, in terms equivalent to those of a solution of 1 gm. per ml. of concentration and 1 cm. path length. The above coefficient can, of course, be converted into terms equivalent to gm. per liter or moles per liter of concentration, by multiplication by suitable constants (10^{-3} and 111.1×10^{-3} respectively, since the molecular weight of cytosine is 111.1; the latter yields a result of 4.83×10^3).

The quartz slides used for evaporated films are carefully cleaned and rendered hydrophilic by thorough scouring with Shamva.⁸ At the 10 cm. filament to slide distance employed, the copper template restricts the film to an area within which the film thickness is uniform to within 1 per cent or less. With such a uniform film, spectra taken at various temperatures may be compared directly, even though different portions of the film may have been used for the different spectrograms.

The evaporated film technique is suitable for the study of purines, pyrimidines, and amino acids, but it cannot be applied to highly polar substances of high molecular weight that decompose before developing even a small vapor pressure. For instance, it was possible to sublime adenosine in a high vacuum, but not adenylic acid. Non-polar substances of low or moderate molecular weight sublime readily, but, instead of depositing as clear homogeneous films, they tend to form crystals that scatter too much light to permit absorption measurements. Similar phenomena had been noted by Hausser *et al.* (35). No improvement was obtained by treating the slides with siloxane to render them hydrophobic, and it has not been possible thus far to apply the evaporated film technique to non-polar compounds.

The absorption spectra of some evaporated film preparations (*e.g.* uracil) are observed to undergo marked changes as a function of age when the films are stored in an atmosphere containing water vapor (36). This effect must be taken into account in interpretations of spectra.

⁸ A metallographic polishing agent obtained from the Golwynne Magnesite and Magnesia Corporation, 420 Lexington Avenue, New York.

Films of polar compounds of high molecular weight such as nucleotides, nucleic acids, and some proteins have been prepared by gradually evaporating an aqueous solution onto a hydrophylic quartz surface. These films were somewhat non-uniform and showed drying rings, but were generally quite clear and satisfactory for spectroscopy, except in the case of some of the protein films. Attempts to prepare dried films of non-polar substances on hydrophobic surfaces were unsuccessful.

Usable films of substances of low melting point were prepared by fusion of the material between two slides, followed by rapid chilling, although light scattering by these specimens was often greater than might be desired.

Most biochemical substances can be investigated by at least one of the above methods of sample preparation. Effects of pH may be studied in the more polar mixtures such as glycerol-water. However, there is need for further investigation of sample preparation in order to extend the range of conditions for low temperature biochemical spectroscopy; a wider variety of solvent mixtures suitable for use at 77 °K. and below is greatly needed. With microspectroscopic techniques (37), single crystals may be employed; this method is of special interest for comparing the spectral detail at reduced temperatures in well oriented specimens on the one hand and random molecular aggregates on the other.

Spectroscopic Methods

To reveal all the fine structures in low temperature absorption spectra, intensity measurements should be obtained as a continuous function of wave-length. This requires either spectrography or continuously recording photoelectric spectrophotometry rather than point by point measurements, as made with such instruments as the model DU Beckman spectrophotometer. The effective resolution of the dispersing device should correspond to a half intensity spectral band width considerably less than the minimum wave-length separation to be observed. Bands 10 Å apart or less have been found near 2600 Å in our low temperature spectra, whereas ordinary photoelectric spectrophotometers usually require half intensity band widths much greater than this for the satisfactory measurement of high densities in this region. Hence spectrographic recording is the only feasible method for quantitative low temperature spectroscopy unless a specially designed recording photoelectric spectrophotometer of unusually high effective resolution can be used. Although we have available appropriate photoelectric equipment (38), we have employed spectrographic methods for all of the low temperature work presently reported because of their technical simplicity and adaptability to use in other laboratories.

Room temperature spectra of all samples were determined with a Beck-

man model DU spectrophotometer as well as spectrographically, as a preliminary step both to insure that the optical densities of the specimens were appropriate and to verify the spectrophotographic results.

Three spectrographs were used: the 35 foot Wadsworth-mounted concave grating of the Massachusetts Institute of Technology Spectroscopy Laboratory, a Bausch and Lomb medium quartz spectrograph, and a Hilger quartz spectrograph model E-1. The latter, which has an effective resolution of approximately 1 Å at 2500 Å with the slit widths employed (20 to 30 μ), was found entirely adequate for survey work and has been used routinely for this purpose, but spectrographs of higher dispersion, such as the others mentioned, are preferable when the greatest accuracy of wave-length determinations is desired. Eastman spectrum analysis type II plates were the most generally useful of those tested for low temperature work and permitted measurements to about 2250 Å with the usual exposure conditions; spectrum analysis type I and Eastman contrast lantern slide plates were also satisfactory.

It is essential to use a light source that yields a continuous spectrum. We have used Hanovia capillary hydrogen discharge tubes, which afford sufficient intensity for reasonable exposure times in most low temperature work when operated at 2500 volts, 100 to 250 ma. A higher powered hydrogen arc (approximately 1 kilowatt discharge through a 3 mm. cylindrical orifice 5.0 mm. long) developed in our laboratory (39) is better suited for applications such as microspectroscopy in which greater total radiant power and larger angular aperture are desirable.

The positions of bands in low temperature spectra may be located by visual examination of spectrograms, as in the work of Bawden, Lavin, and others (see, for example, (14-17)) or by direct visual observation of spectra in the visible region, as in the work of Keilin and Hartree (24). For more precise wave-length determinations and semiquantitative indications of relative densities, the spectrophotographic recording technique of Holiday (40), used also by Brown and Randall (23), is convenient. In this method, the photographic plate is moved across the spectrum during exposure by means of a logarithmic cam that varies the time of exposure, so that the developed plate exhibits a pattern of blackening that corresponds roughly to a plot of the absorption spectrum.

If density values as well as wave-length positions are to be determined quantitatively in low temperature spectrography, the plates must be calibrated by suitable comparison exposures (*e.g.*, with a solvent cell or blank slide in the beam). Mayneord and Roe (18) used a Spekker photometer for this purpose, determining densities from the plates by visual matching of the comparison and specimen spectra. We have calibrated our plates by varying the exposure time, after the method of Henri (29, 41), and have measured the plate blackening objectively with a Sinclair

Smith recording microdensitometer. Normally, fourteen comparison spectra (exposure times 5 to 50 seconds) are photographed on the same plate to permit ease of interpolation and multiple checks in determining densities. Exposures are either controlled manually and timed by a stop-watch or controlled automatically by means of an integrating photoelectric circuit that operates the shutter. Fig. 3 shows a portion of a spectrum plate and Fig. 4 shows a typical microdensitometer record.

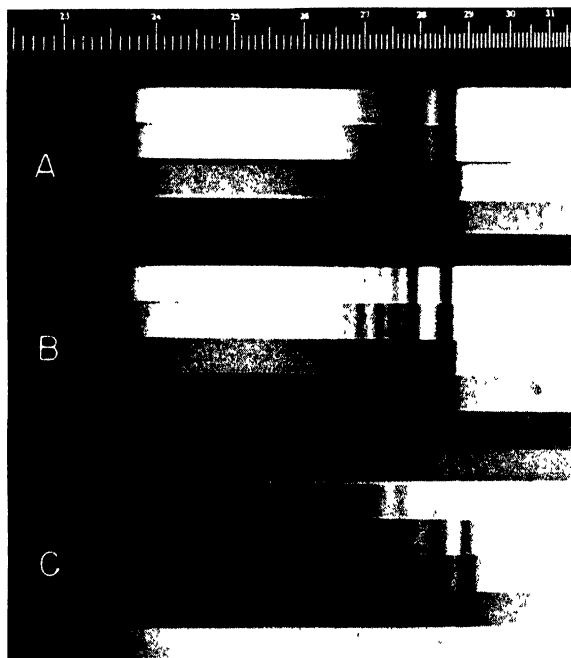


FIG. 3. Portion of a typical low temperature spectrogram (77 °K.) of solid film preparation. A, *p*-methoxyphenylalanine; B, *p*-hydroxyphenylalanine; C, cytosine. Most of the comparison spectra are not shown. The numbers on the wave-length scale represent angstroms in hundreds.

In computing densities from the microdensitometer traces, it is assumed that, for equal blackening by two exposures at a particular wave-length, the intensities are inversely proportional to the exposure times; *i.e.*, it is assumed that the reciprocity law holds. As normally used, all emulsions deviate more or less from this law. Under our experimental conditions, the reciprocity error was found to be of the same order of magnitude as other errors, and the use of other means of controlling exposure (*e.g.*, a rotating sector) was therefore not thought to be warranted.

Relative values of optical density at different wave-lengths may be

determined by the above method with an over-all average error of 2 or 3 per cent (22). Repeated measurements and comparisons with data obtained with the Beckman spectrophotometer indicate that the shapes of absorption curves are quite accurately and reproducibly determined. The over-all error in the determination of absolute densities may be considerably greater, averaging perhaps 5 per cent and ranging up to 15 per cent in unfavorable instances, especially if there is appreciable light scattering by the sample. Fig. 5 illustrates density *versus* wave-length plots for spectra determined at 298° and 77 °K. by this method.

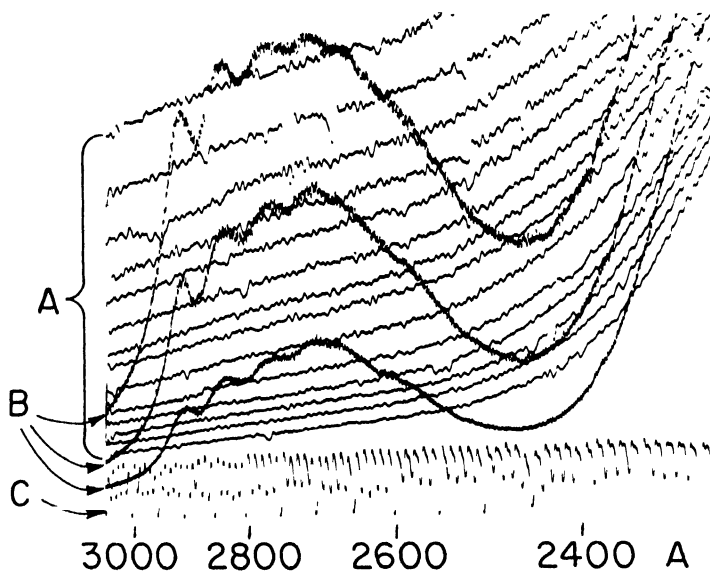


Fig. 4 Typical microdensitometer record. A, traces of control spectra, B, traces of absorption spectra, C, trace of wave-length scale

Handling of Specimens

At room temperature, the quartz slide or cell is placed in front of the spectrograph slit and is set back some distance if the specimen is non-uniform, so that the defects will not be imaged in the spectrum. Collimated light is obtained by placing a diaphragm immediately in front of the long, thin hydrogen discharge tube and using the center hole of the Hartmann diaphragm over the spectrograph slit.

At reduced temperatures, a quartz Dewar vessel with two sets of opposing plane fused quartz windows (Fig. 6) is used (42). For work at 77 °K., the slide or cell is placed directly in the liquid nitrogen and the light beam is sent through the Dewar windows, liquid nitrogen, and

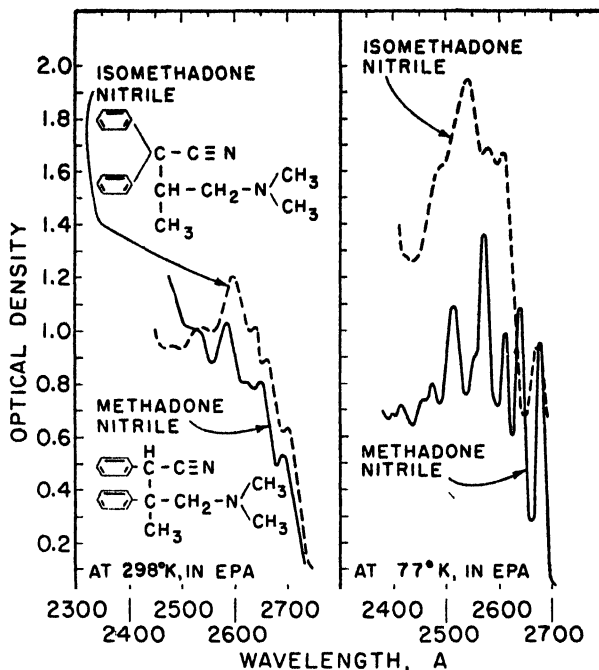


Fig. 5. Absorption spectra of methadone nitrile and isomethadone nitrile determined by photographic spectrophotometry at 298° and 77°K. in EPA solvent. The actual measured densities are shown for solutions of the same molar concentration since relative densities are determined more accurately by the method than are absolute absorption coefficients. As illustrated, there is marked difference in the effects of low temperatures on the spectra of these two isomers.

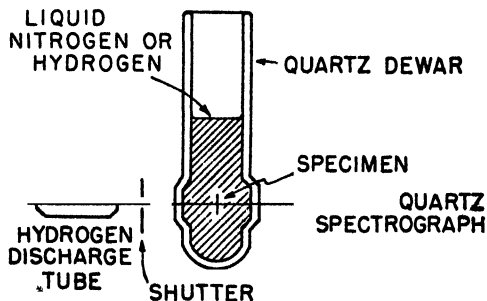


Fig. 6. Set-up for absorption spectroscopy at 77°K.

specimen. Special plastic or cork holders that rest on the bottom of the Dewar vessel are used for slides, whereas cells are suspended from above. After temperature equilibrium has been attained, no serious difficulty is

encountered from the formation of gas bubbles in the nitrogen. In the case of isopentane mixtures, about 7 minutes immersion time is required for complete vitrification and attainment of liquid nitrogen temperature with the cells we have employed. Since liquid oxygen has strong absorption bands in the near ultraviolet, it is essential that only very pure liquid nitrogen be used in the light path; Linde Air Products "high purity" nitrogen is satisfactory, provided it is not allowed to stand in contact with air.

For work at 21 °K. with liquid hydrogen appropriate precautions must be taken to prevent explosions. Furthermore, air must not be allowed to enter the Dewar vessel; otherwise, the oxygen and nitrogen will freeze and fall through the liquid hydrogen, frosting the windows and specimens. To prevent this difficulty, a special glass cap was fitted snugly into the top of the Dewar flask against a cushion of glass wool. One of the two inlet tubes of the cap was stuffed with glass wool and served for the gradual release of hydrogen. The cap contained four slides in thin plastic holders that could be lowered through the liquid into the light beam and raised again, after the manner of a puppet show, by means of silk threads that passed through holes in a brass plug on the second inlet tube. The slide holders were prevented from twisting by small, soft iron bars attached to their bottoms, which responded to the field of an external aligning magnet. With one blank slide and three specimens, it was possible to obtain three spectra with one charge of hydrogen. Air present at the time of filling was completely flushed out by the hydrogen vaporized in cooling the Dewar walls. As a result of small leaks, it was impossible to prevent gradual internal frosting of the windows while the spectra were being taken. Consequently, we have not attempted to determine absolute optical densities at liquid hydrogen temperature; the relative densities and absorption curve shapes are accurate and reproducible, as in the case of spectra taken at liquid nitrogen temperature.

We have designed and tested a double Dewar vessel for use at 4 °K. However, we have encountered such difficulty from internal frosting as a result of air leaks during the spectrum exposures as to require redesigning of the equipment before satisfactory spectra can be taken at liquid helium temperature.

It is necessary to use greater light beam intensities for equivalent exposure times in low temperature as compared with room temperature spectrography because of light losses at the Dewar windows, and light scattering by bubbles in the cooling solution (especially in liquid hydrogen), etc. On humid days, moisture condensation on the outer windows of the Dewar flask may be prevented by directing an air blast against them.

If the quartz slides bearing film preparations are removed from the Dewar vessel directly into the laboratory atmosphere, moisture condenses on them and dissolves the films. The slides are therefore plunged into a non-polar solvent of low boiling point (anhydrous ether or hexane) immediately upon withdrawal from the flask. After the slides have come to room temperature, they are gradually removed from the liquid, which rapidly evaporates from their surfaces; the slides may then be preserved for further use.

SUMMARY

1. Theoretical considerations and limited experimental evidence suggest that at greatly reduced temperatures (77–74 °K.) it should be possible to observe spectroscopic fine structure in visible and ultraviolet absorption spectra. This fine structure represents vibrational and rotational transitions associated with the primary electronic transitions giving rise to absorption bands. Such increased detail in spectra should be useful for the identification and analysis of complex substances and for molecular structure studies. Other phenomena associated with low temperature spectroscopy are of interest in studying energy transfer between molecular groupings.

2. Various techniques for the preparation of specimens for low temperature spectroscopy are described. In two methods vitrified solvents are used, ether-isopentane-alcohol mixtures or glycerol-water mixtures. In a third method, thin films are prepared by sublimation in a high vacuum. Thin films may also be prepared in some instances by precipitation from an evaporating solvent or by melting of the desired substance, followed by rapid chilling.

3. Spectrophotometric techniques that have been found useful for low temperature spectroscopy are described in detail.

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ULTRAVIOLET ABSORPTION SPECTRA AT REDUCED TEMPERATURES

II. PYRIMIDINES AND PURINES*

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The strong ultraviolet absorption bands of pyrimidines, purines, and their derivatives between 2400 and 2800 Å (1) have been used widely for the characterization and quantitative determination of these biologically important compounds, and the 2600 Å band, attributable to the presence therein of these substances, has been employed extensively for the cytochemical localization of nucleic acids (2). The broadness of the absorption bands at room temperatures leads to considerable lack of specificity; the observation of greater spectral detail is therefore highly desirable.¹ Furthermore, the increased information about molecular structure yielded by spectra that show vibrational fine structure would be useful in studying molecular rearrangements, molecular interactions, and stereochemical and energy transfer problems of interest in relation to this series of compounds (3-7). Accordingly, naturally occurring pyrimidines and purines and derivatives thereof were studied by the techniques of low temperature spectroscopy described in the first paper of this series (8).

Materials and Methods

Thymine, cytosine, adenine, and guanine were used as obtained from the Schwarz Laboratories. Counter-current distribution studies according to the method of Williamson and Craig (9) indicated that these compounds contained less than 5 per cent of ultraviolet-absorbing impurities. Schwarz uracil was recrystallized four times from water to constant absorption coefficient (molecular absorption coefficient, $\epsilon_{250} = 8030$ at pH 7.0), constant maximum to minimum ratio ($\epsilon_{250}/\epsilon_{230} = 5.45$ at pH 7.0), and constant nitrogen value (24.1 per cent). 1,3-Dimethyluracil was

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¹ This is true even though differences in the spectral response of these substances to pH change and irradiation are useful additional factors in their characterization.

prepared from Schwarz uracil by methylation with diazomethane after the method of Case and Hill (10). The product was recrystallized twice from absolute alcohol to constant absorption coefficient ($\epsilon_{298} = 8515$ at pH 6.5), constant nitrogen content (19.8 per cent), and constant melting point (123–124°, corrected).

5-Methylecytosine, 2-thiothymine, and 2-thiocytosine were used as obtained from the Dougherty Company. The isocytosine employed was prepared from Dougherty isocytosine by a double fractionation in the Craig counter-current apparatus, first at pH 11.0 and again at pH 7.0, to free the material from contamination with cytosine. The product had the characteristic absorption spectra of isocytosine in solutions of various pH values, as reported by Stimson and Reuter (11), and melted at 264°.

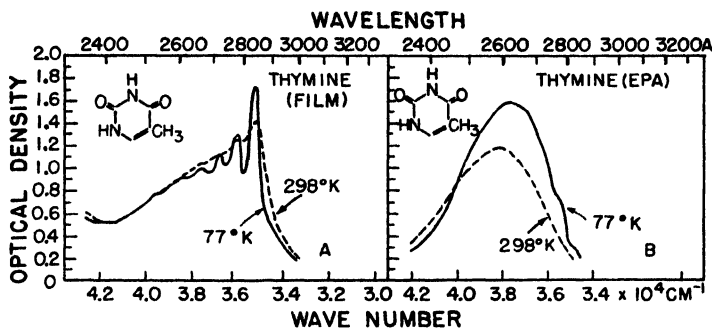


Fig. 1. Absorption spectra of thymine at 298° and 77 °K. A, sublimed film; B, solution in EPA.

2-Thiouracil was used as obtained from the Bios Laboratories, Inc. Isoguanine was obtained through the courtesy of Sister Miriam Michael Stimson and is the material used in her reported investigations (12).

The methods employed for obtaining the spectra and the special solvents and cells used, etc., have been described in detail previously (8).

Results

Thymine—Fig. 1, A and B, indicates the absorption spectra, respectively, of a sublimed film of thymine and of thymine in EPA² solvent at 298 °K. and 77 °K.³ The sharpening of the spectrum is considerably

² Ethyl ether, isopentane, and ethyl alcohol in the proportions of 5:5:2 by volume (see also (8)).

³ The spectra of non-refrigerated samples were taken at room temperature, which averaged about 24–27° and is referred to in the text and figures as 298 °K. The spectra of refrigerated samples were taken (with one exception noted in the text) with the substance in temperature equilibrium with boiling liquid N₂ or H₂, the boiling points of which are respectively 77.4 °K. and 20.5 °K. and (13) are stated in the text and figures as the approximate values, 77 and 21 °K., respectively.

more evident in the film spectra, with an obvious development of subsidiary maxima corresponding to vibrational transitions accompanying the primary electronic transition. Such transitions are represented only as shoulders in the spectrum in the EPA solvent. The transitions appear to represent the fundamental and overtones of a single vibrational frequency at about 740 cm^{-1} , for which an infra-red band has been reported (14).

The general shape of the spectrum of the film resembles that of thymine in aqueous solution at alkaline pH, while that of the spectrum in the EPA solvent is similar to the solution spectrum at neutral or acid pH. This fact suggests that the thymine molecules in the film may be in an enolic form or that there may be bonding between hydrogen atoms attached to one ring and keto groups of an adjacent ring.

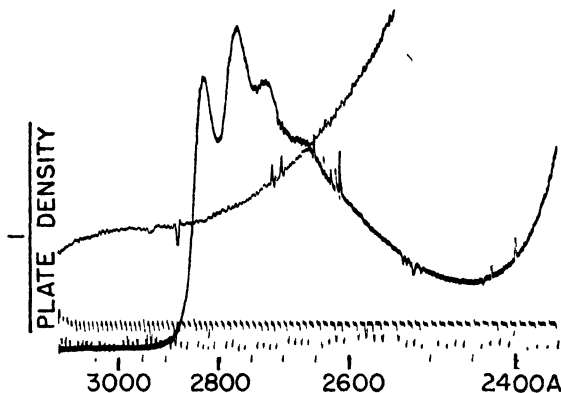


FIG. 2. Microphotometer trace of absorption spectrum of thymine film at 211 °K.

A microphotometer trace of the spectrum⁴ of a thymine film at an intermediate temperature (211 °K.) is shown in Fig. 2. This spectrum is obviously intermediate between those at 298 °K. and 77 °K. Further refrigeration of thymine to 21 °K. did not, however, detectably alter the spectrum from that obtained at 77 °K.

Table I indicates the wave-lengths of the maxima and minima of these spectra and the approximate range of the shoulders within $\pm 3\text{ Å}$.

Uracil—As shown in Fig. 3, the absorption spectra at 77 °K. of sublimed uracil films have been found to change with aging of the films upon standing in the laboratory at room temperature (15). The presence of water vapor is necessary for this effect, which is believed to be due primarily to

⁴ This spectrum was obtained by immersing the film in spectroscopically purified isopentane which had been chilled to dry ice temperature and was used to fill the quartz Dewar flask. The temperature was measured with an immersion pentane thermometer.

TABLE I

Structural Detail of Absorption Spectra at Low Temperatures

77 °K. unless otherwise indicated.

Compound	Maxima	Minima	Shoulders
	λ	λ	λ
Thymine (film)	2830 (2816)* 2770 (2760) 2713 (2712) 2657 (2650)	2792 (2790) 2733 (2730) 2675 (2674) 2383	2622-2610 2573-2559 2534-2506
“ (EPA)	2654		2884-2856 2817-2797 2699-2667
Uracil (film; 2 days) . . .	2863 2793 2727 2677	2849 2778 2711 2388	2662-2637 2585-2554
“ (EPA)	2694 2643 2615	2686 2626	2823-2799 2754-2742 2566-2556 2504-2494
1,3-Dimethyluracil (EPA)	2670		2836-2807 2722-2691 2654-2628
Cytosine (film)	2900 (2898) 2837 (2831) 2766 (2769) 2655 (2655) 2602 2558	2874 (2875) 2806 (2804) 2743 (2744) 2632 2576 2447	2707-2685 (2703-2682) 2507-2470 2470-2447
“ “ 21 °K.	2902 2889 2838 2828 2763 2742 2657 2598 2559	2897 2871 2834 2801 2746 2738 2637 2578 2438	2911 2871-2863 2863-2849 2709-2685 2526-2487 2487-2438
“ (EPA)	2859 2740 2694 2493	2844 2722 2513 2478	2805-2788 2675-2649 2615-2595 2575-2549 2539-2519
Isocytosine (film)	3042 2977 2794 2689	3028 2836 2777 2514	3134-3103 2958-2926

TABLE I—*Concluded*

Compound	Maxima	Minima	Shoulders
	<i>A</i>	<i>A</i>	<i>A</i>
Isocytosine (EPA)	2894 2774 2701	2793 2731 2460	3044-3002 2956-2935 2854-2834 2677-2656 2626-2601 2577-2549
5-Methylcytosine (EPA) .	2870 2761	2860 2564	2935-2913 2815-2794 2732-2717 2686-2955
Thiouracil (film)	3164 3063 2767	3098 3024 2667-2637	
“ (EPA)	2717	2415	2976-2873
Thiothymine (film)	3062-3012 2533	2869 2398	3309-3279 3174 3136-3098 3028-2844
“ (EPA)	2760	2436	
Thiocytosine “	3144 2992 2835	3043 2972 2671	
Adenine (film; 3 days)	2778 2723 2686	2752 2711 2360	2882-2864 2835-2810
Guanine (film)	2784 2473	2673 2320	
Isoguanine (film)	2912 2388	2621 2330	

* The data in parentheses are from spectra obtained with a Wadsworth-mounted 35 foot grating spectrograph.

aggregation of the uracil molecules, in the presence of a thin film of water, into a crystalline mosaic.

Fig. 4, *A*, presents the spectra of uracil in EPA. The 77 °K. spectrum is generally similar to that of thymine in EPA but the vibrational bands are much more pronounced and the whole spectrum is shifted toward shorter wave-lengths.

1,3-Dimethyluracil—The spectra of this compound in EPA are given in Fig. 4, *B*. The addition of the two methyl groups appears to have severely damped the vibrations, thus leading to shorter life time in the excited state and loss of vibrational detail as compared with the corresponding spectra of uracil (Fig. 4, *A*), in accordance with the principles discussed in the preceding paper (8).

Cytosine—The low temperature (77 °K.) absorption spectra of cytosine exhibit a wealth of vibrational detail as shown in Fig. 5, *A* and *B*, of a sublimed film and an EPA solution, respectively. The vibrational struc-

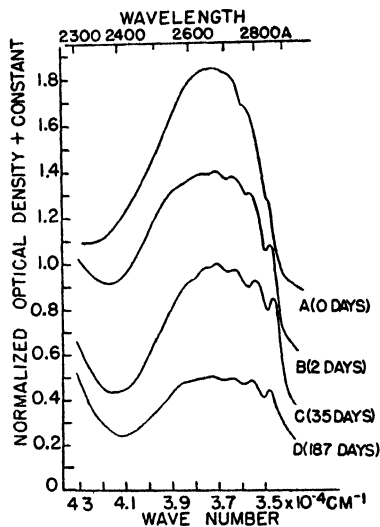


FIG. 3. Uracil, sublimed films. Absorption spectra at 77 °K. at the indicated times after sublimation. Optical densities normalized to 1 at the absorption maximum and separated by added constants. The respective normalizing fractions and added constants are (Curve A) $\times 0.73$, $+ 0.85$; (Curve B) $\times 1.1$, $+ 0.4$; (Curve C) $\times 0.54$, $+ 0$; (Curve D) $\times 0.69$, $- 0.5$.

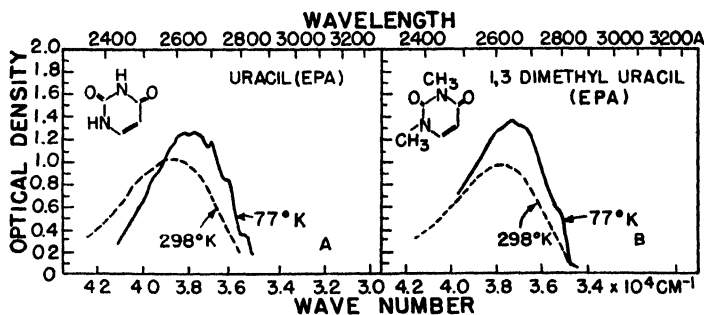


FIG. 4. *A*, absorption spectra of uracil at 298° and 77 °K. in EPA; *B*, absorption spectra of 1,3-dimethyluracil at 298° and 77 °K. in EPA.

tures of the spectra of the film and of the solution in EPA are in general very similar, with, however, considerable differences in the relative intensities of the various subsidiary maxima. The spectrum in EPA is shifted toward shorter wave-lengths as compared to that of the film.

At 21 °K., an even greater resolution of the film spectra vibrational bands is obtained, as shown by the microphotometer trace, Fig. 6, and the data in Table I (see also (8) Fig. 3).

As with thymine, the major vibrational bands in the 77 °K. spectrum appear to be the fundamental and harmonics of a single vibrational frequency, in this case 810 cm^{-1} . That these are, however, actually com-

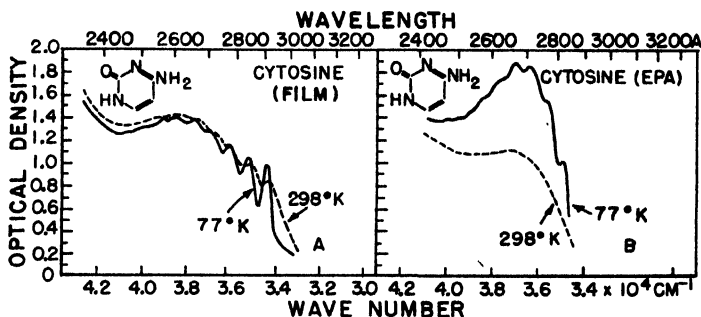


Fig. 5. Absorption spectra of cytosine at 298° and 77 °K. A, sublimed film; B EPA solution.

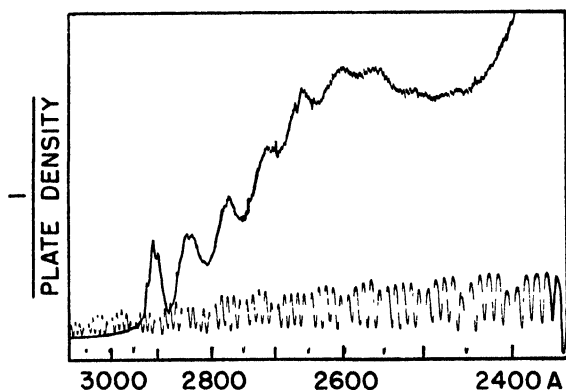


Fig. 6. Microdensitometer trace of the absorption spectrum of a sublimed film of cytosine at 21 °K.

posite bands is revealed by the spectrum at 21 °K. A strong infra-red band has been reported at about 800 cm^{-1} (16).

Isocytosine—The 77 °K. spectra of isocytosine as a sublimed film and in the EPA solvent (Fig. 7, A, B) are considerably less detailed than those of cytosine. The widths of the peaks and shoulders of these spectra make it difficult to compare their vibrational frequencies with those of cytosine.

5-Methylcytosine—The EPA spectrum of this compound, which bears

the same relation to cytosine as does thymine to uracil, are shown in Fig. 8. The spectral relations parallel this chemical relation as there is less structure in the 5-methylcytosine EPA spectrum than in that of cytosine, and the former spectrum is displaced toward longer wavelengths.

2-Thiouracil—The spectra of the thiosubstituted pyrimidines appear to possess a second absorption peak in the region of 3100 to 3400 Å (17). The spectra at 298° and 77 °K. of thiouracil as a sublimed film and in

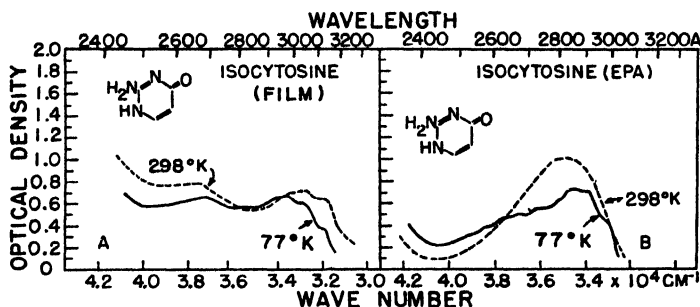


FIG. 7. Absorption spectra of isocytosine at 298° and 77 °K. A, sublimed film; B, EPA solution.

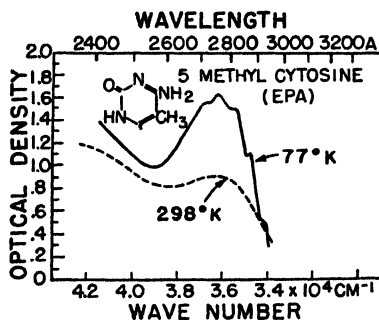


FIG. 8. Absorption spectra of 5-methylcytosine in EPA at 298° and 77 °K.

EPA are presented in Fig. 9, A and B. In the film, the 77 °K. spectrum develops a very intense maximum at the 3164 Å peak, with two subsidiary maxima, whereas no vibrational structure is developed at the 2769 Å peak in the usual pyrimidine absorption region. The spectrum of the EPA solution shows less change upon refrigeration than does that of the sublimed film.

2-Thiothymine—Absorption spectra of a sublimed film and an EPA solution of this compound are shown in Fig. 10, A and B, respectively. In the film, the vibrational structure developed near the 3042 Å peak is

considerably less at 77 °K. than is the case for thiouracil; no fine structure appears at the 2533 Å peak.

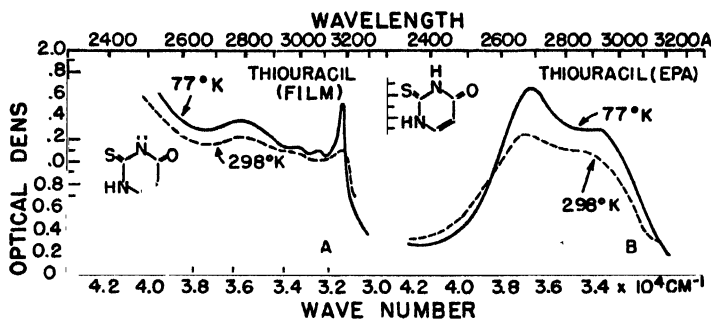


Fig. 9. Absorption spectra of thiouracil at 298° and 77 °K. A, sublimed film; B, EPA solution.

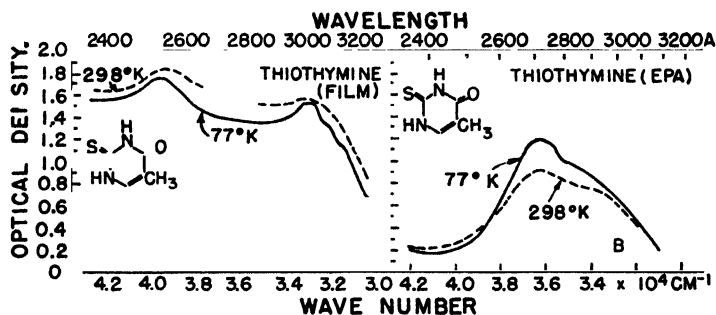


Fig. 10. Absorption spectra of thiothymine at 298° and 77 °K. A, sublimed film; B, EPA solution.

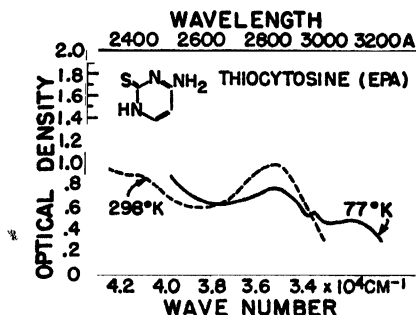


Fig. 11. Absorption spectra of thiocytosine in EPA at 298° and 77 °K.

2-Thiocytosine—The EPA solution of 2-thiocytosine provides the spectra shown in Fig. 11. At 77 °K, three distinct absorption maxima are discernible,

Adenine—As with uracil, the low temperature absorption spectra of sublimed films of this compound have been found to change with aging of the films upon standing in the laboratory at room temperature. The spectra of a freshly sublimed film are shown in Fig. 12, A, while those of a film 3 days after sublimation are shown in Fig. 12, B. As can be seen, there is a limited development of vibrational structure upon standing. The relative distribution of intensity among the five vibrational peaks

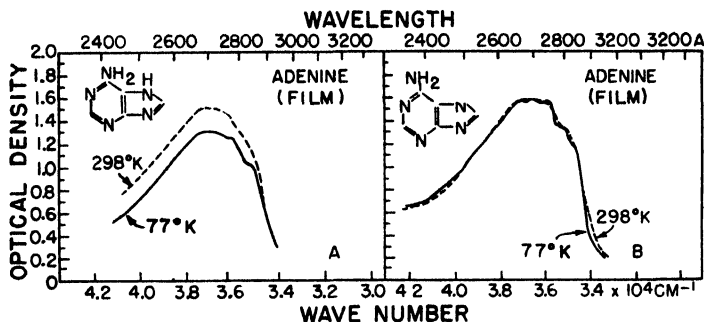


Fig. 12. Absorption spectra of sublimed films of adenine at 298° and 77°K., showing the effect of standing. A, spectra taken immediately after preparation; B, spectra taken 3 days later.

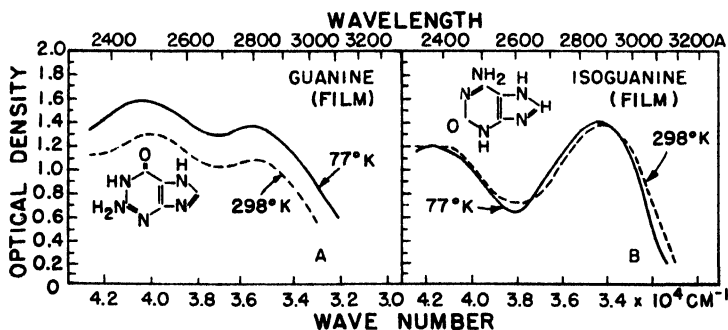


Fig. 13. A, absorption spectra of a sublimed film of guanine at 298° and 77°K.; B, absorption spectra of a sublimed film of isoguanine at 298° and 77°K.

has been found to vary in different films for undetermined reasons. Further refrigeration to 21°K. did not influence the film spectrum.

Guanine—The 77°K. spectrum of this purine is essentially identical with its spectrum at 298°K. (Fig. 13, A). There have been some indications of a development of a very limited vibrational structure in aged

(6 months) films of guanine, but this subject requires further investigation.⁵ Refrigeration to 21 °K. did not influence the guanine spectrum further.

Isoguanine—Here again, as shown in Fig. 13, *B*, there is very little alteration of the spectrum upon refrigeration of a sublimed film.

Xanthine and Hypoxanthine—As with guanine and isoguanine, refrigeration of sublimed films of these materials had little effect upon their absorption spectra.

DISCUSSION

It appears that in many cases there is a marked increase in detail in the low temperature absorption spectra of the various pyrimidine compounds studied, with a consequent unveiling of the vibrational structure accompanying the electronic transition responsible for the absorption. The degree of sharpening obtained varies markedly among the various compounds and depends upon the environment of the absorbing material; sublimed films usually provide more detailed fine structure than do solutions in EPA.

The addition of methyl groups to the pyrimidine ring appears to damp the vibrations and reduce the spectral detail obtained, at least in EPA solutions, as evidenced by thymine, 1,3-dimethyluracil, 5-methylcytosine, and 2-thiothymine.

Substitution of a keto group by a thio group in uracil, thymine, and cytosine results in the development of a new absorption band at longer wave-length. Any vibrational fine structure developed in the spectra at low temperature appears to be confined to this band, while the usual pyrimidine band is resistant to the effect of low temperature.

The addition of the fused imidazole ring to the pyrimidine nucleus, as in the purines, appears to damp the upper electronic state and to reduce greatly the fine structure obtained in the low temperature spectra (compare isoguanine and cytosine). Low temperature spectroscopy thus appears to be particularly useful for the characterization and differentiation of the various members of the pyrimidine series rather than for members of the purine group. Differences in the effects of temperature upon pyrimidines and purines may be of use in differentiation between the two. Such differences are also of interest because of the possibilities they suggest of energy transfer between condensed rings in this series.

⁵ Brown and Randall (18) have reported the presence of five bands in the low temperature spectra of a sublimed film of guanine but we have been unable to confirm their observation.

SUMMARY

1. The absorption spectra, at 298 °K., 77 °K., and in some instances 21 °K., of a variety of pyrimidine and purine compounds are presented and discussed.

2. In general, low temperature absorption spectra of the pyrimidines exhibit considerable sharpening with development of subsidiary bands corresponding to vibrational transitions, whereas low temperature absorption spectra of the purines are but little changed from those at room temperature.

3. Low temperature spectroscopy appears to offer a useful tool for the characterization and differentiation of variously substituted pyrimidines. It also yields data of interest from the standpoint of the energy states in molecules of this series.

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THE MECHANISM BY WHICH GLYCERALDEHYDE INHIBITS GLYCOLYSIS*

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In 1929 Mendel (1) discovered that DL-glyceraldehyde in concentrations of 10^{-3} M almost completely inhibited the anaerobic formation of lactic acid from glucose by rat sarcoma. Glyceraldehyde, even in much higher concentrations, did not appreciably affect the respiration of normal liver, kidney, and brain, or of malignant cells. Somewhat later Mendel, Bauch, and Strelitz (2) found that the addition of small amounts of pyruvate reduced the inhibitory action of glyceraldehyde on glycolysis of tumor slices. The inhibition of glycolysis by glyceraldehyde was confirmed by Ashford (3) and Holmes (4) with brain tissue, by Needham and Nowinski (5) with chick embryos, and by many other investigators using a variety of tissue preparations (6-15). These workers have established the conditions under which glyceraldehyde inhibition may be obtained and have uncovered several important clues regarding the site of its action but the mechanism by which this compound inhibits glycolysis has remained obscure.

While glyceraldehyde strongly inhibits lactate formation when glucose is the substrate, it is almost completely without influence on the breakdown of starch or glycogen to lactic acid (4, 6, 11). The glyceraldehyde inhibition of glycogen breakdown in muscle extract reported by Boyland and Boyland (12) and Adler *et al.* (9) was later explained by the results obtained by Lehmann and Needham (13). The latter workers found the dimeric form of glyceraldehyde, which exists in fresh solutions, to inhibit the conversion of glycogen to the Cori ester by phosphorylase in the same manner as do glucose and mannose (16). The monomeric form does not inhibit the conversion of glycogen to hexose monophosphate (HMP) (16)

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nor does it prevent lactate formation from HMP (17-19) or hexose diphosphate (HDP) (7, 20).

It thus appears that glyceraldehyde exerts its influence at the first stage of sugar metabolism; namely, the phosphorylation of hexose by adenosinetriphosphate (ATP). This consideration led Stickland (14) to reinvestigate the effect of glyceraldehyde on glucose breakdown by muscle extract in the presence of yeast hexokinase (4, 6, 17, 18). He found that small excesses of hexokinase (above that required to obtain moderately rapid utilization of glucose by muscle extract) were very effective in preventing inhibition of glycolysis by glyceraldehyde.

While these results point to hexokinase as the site of action of glyceraldehyde, Adler, Calvet, and Günther (9) reported that the latter compound did not inhibit yeast hexokinase prepared according to Meyerhof (21). More recently Rudney (22) has reported that glyceraldehyde inhibits the hexokinase activity of yeast, beef brain, rat tumor, and rat skeletal muscle.

The fact that glyceraldehyde has no effect on glycolysis of polysaccharides or phosphorylated sugars in muscle extracts, yet strongly inhibits glycolysis in tumor, embryo, and brain, has been cited (6, 7, 11, 23, 24) as evidence in favor of a non-phosphorylating glycolysis in the latter tissues. Needham and coworkers (13, 24) contend that the condensation, in muscle extract, of dihydroxyacetone phosphate (a breakdown product of HDP) with glyceraldehyde (25) removes the latter from solution and thus prevents it from inhibiting glycolysis. They maintain that since glyceraldehyde is not similarly removed by embryo, tumor, and brain HDP cannot be an intermediate in glycolysis by these tissues. It is not the purpose of the present paper to add to the overwhelming evidence establishing the occurrence of phosphorylating glycolysis in all of these tissues (26-29). None the less, the mechanism of glyceraldehyde inhibition established by this investigation is the very antithesis of the non-phosphorylating glycolysis hypothesis. It will be shown that glyceraldehyde inhibits glycolysis only as a consequence of its condensation with dihydroxyacetone phosphate.

Methods and Materials

Hexokinase activity was measured by the manometric technique of Colowick and Kalckar (30) with 95 per cent N_2 and 5 per cent CO_2 as the gas phase. A dialyzed and lyophilized extract of beef brain was used as the enzyme source. This was prepared from the acetone powder of Colowick, Cori, and Slein (31), as described by Wiebelhaus and Lardy (32). From 7 to 12.5 mg. of this preparation were used per flask (final fluid volume 3.0 ml.), depending on the activity. Experiments on animal

hexokinases were all carried out at 37°, those on yeast hexokinase at 30°. The reaction mixture, expressed in final molarities, was as follows: K-ATP 0.004 M (side arm), KHCO₃ 0.027 M, K iodoacetate 0.002 M, KF 0.053 M, and MgCl₂ 0.008 M. Any variations from the above mixture will be pointed out. Unless otherwise specified, 0.01 M glucose was the substrate. Details of the experimental procedure have been described (32). All the values reported are corrected for the CO₂ liberation measured in a flask containing ATP and the other constituents but no substrate. This small blank presumably results from the enzymatic hydrolysis of ATP or of minor contaminants. By this method the sum of hexokinase and phosphohexokinase activities when free hexoses are used as substrates are measured, since both of these enzymes are present in the brain preparation (32). Phosphohexokinase activity was measured by using glucose-6-phosphate or fructose-6-phosphate as the substrate.¹

Phosphorus was determined by the colorimetric method of Fiske and Subbarow as modified by King (33). Triose phosphate was hydrolyzed by treatment of neutralized trichloroacetic acid filtrates with 1 N NaOH at room temperature for 20 minutes. Other phosphate esters were fractionated by the procedure outlined by LePage (34).

DL-Glyceraldehyde was a gift of Professor H. O. L. Fischer and Professor Erich Baer. It was purified by refluxing with acetone as described by Baer and Fischer (35). D- and L-glyceraldehyde were prepared from the 1,2:3,4-diisopropylidene derivatives of their respective mannitols by the methods of Baer and Fischer (36).

Glucose-6-phosphate was synthesized by the method of Lardy and Fischer (37). Fructose-6-phosphate was prepared from HDP by the method of Neuberg, Lustig, and Rothenberg (38). Phosphoric esters of L-sorbose were synthesized by procedures described in the following paper (39).

Results

Our first objective was to determine whether or not brain hexokinase is inhibited by DL-glyceraldehyde. While results with fresh tissue homogenates were variable, a consistent inhibition was obtained with the dialyzed and lyophilized extract of beef brain acetone powder. Concentrations of 7×10^{-4} to 3.3×10^{-3} M DL-glyceraldehyde caused 20 to 90 per cent inhibition. In experiments in which 3.3×10^{-3} M glyceraldehyde produced 40 to 50 per cent inhibition, much higher concentrations of the inhibitor produced only a slightly greater inhibition. Different prepara-

¹ The brain preparation contains all of the enzymes involved in the conversion of glucose to lactic acid. Rapid glycolysis occurs if fluoride and iodoacetate are omitted and the necessary substrates and cofactors are added.

tions of dialyzed and lyophilized brain extract varied greatly in their sensitivity to glyceraldehyde. It was later learned that if the water extracts of the dehydrated brain powder were centrifuged at high speed until free from particulate matter the resulting hexokinase preparations were more sensitive to glyceraldehyde inhibition.

The results in Fig. 1 demonstrate the influence of various concentrations of D-, L-, and DL-glyceraldehyde on the phosphorylation of glucose by a "particle-free" brain preparation. The inhibitory effect of DL-glyceralde-

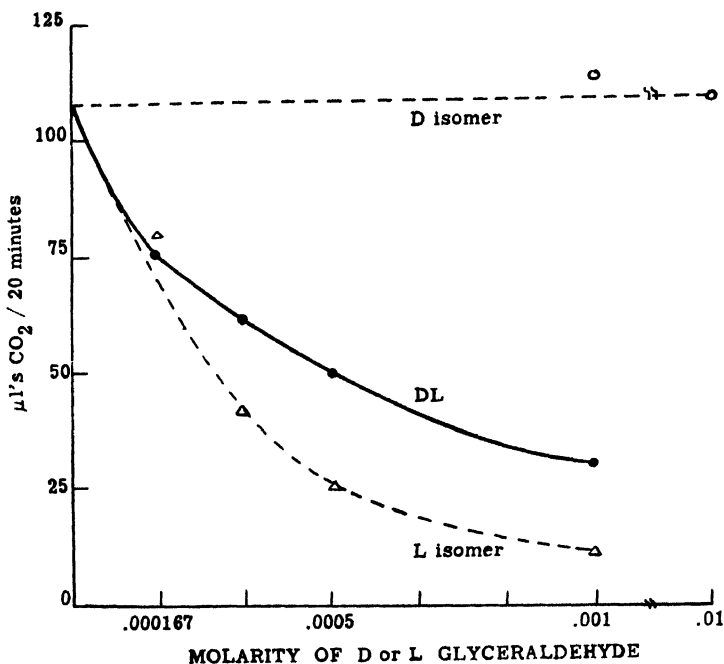


FIG. 1. Effect of concentration of D-, DL-, and L-glyceraldehyde on brain hexokinase activity. Glucose, 0.01 M; ATP, 0.0026 M. The solid line is plotted for molarity of individual enantiomorphs.

hyde can be ascribed entirely to the L component. This is in agreement with the conclusions of Needham and Lehmann (7) and Mendel *et al.* (40). The D isomer failed to show significant inhibition even when tested at 0.05 M. Needham and Lehmann (7) reported that solutions of D-glyceraldehyde, whose rotation had changed from $[\alpha]_D = +14^\circ$ to $+8^\circ$ on standing, were inhibitory to glycolysis in embryonic tissue. They assumed the change in rotation to result from racemization and the inhibition to result from the L component thus formed. However, Meyerhof

et al. (25) had previously demonstrated that the decrease in rotation is not accompanied by racemization and Baer and Fischer (36) have shown that it is probably a mutarotation. In the present study, brain hexokinase was not inhibited by D-glyceraldehyde which had been in solution for many days.

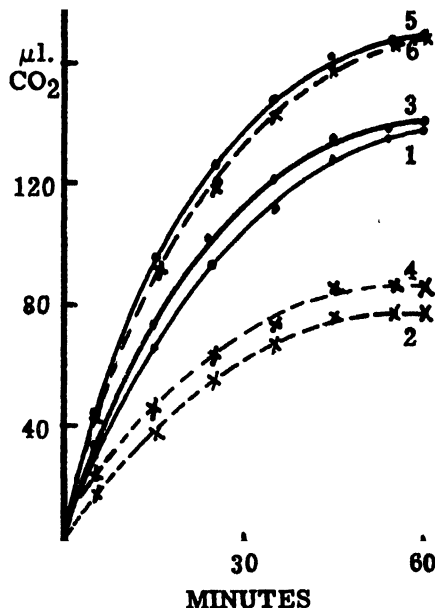


FIG. 2. Substrate specificity to glyceraldehyde inhibition. The solid lines represent control flasks without glyceraldehyde; broken lines, flasks with 3.7×10^{-3} M D-L-glyceraldehyde. Substrates, Flasks 1 and 2, 0.01 M glucose; Flasks 3 and 4, 0.01 M fructose; Flasks 5 and 6, 0.0083 M glucose-6-phosphate or 0.0083 M fructose-6-phosphate (phosphorylation rates with the latter two substrates were practically identical).

From Fig. 1 it appears that L-glyceraldehyde present in the racemate is less effective as an inhibitor than the synthetic (36) L isomer.² A possible explanation will be discussed later.

To determine whether the inhibition observed in our stable brain preparations occurred at the hexokinase or phosphohexokinase stage, the effect of glyceraldehyde was tested on various substrates. As shown in Fig. 2, glyceraldehyde inhibited the phosphorylation of both glucose and fructose, but not of glucose-6-phosphate or fructose-6-phosphate. Thus

² L-Glyceraldehyde synthesized by a different route is equally inhibitory to brain hexokinase (41).

neither phosphohexokinase nor phosphohexoisomerase is inhibited. Separate enzymes seem to be involved in the phosphorylation of glucose and fructose in muscle (42) and preliminary evidence (32) indicated the same to hold for brain. However, we have been unable to demonstrate additive effects from the substrate with combinations of glucose, mannose, and fructose. Hence, it is concluded that the presence of glyceraldehyde leads to inhibition of hexokinase acting on any one of its substrates.

In an attempt to elucidate the nature of the effect of glyceraldehyde, experiments were performed to determine whether the inhibition was of the substrate competitive type. It appeared not to be, for the degree of inhibition caused by 0.0018 M DL-glyceraldehyde was not influenced by varying the concentration of glucose over the limited range of 0.001 to 0.035 M. Varying the concentration of ATP from 0.001 to 0.006 M likewise seemed not to influence the inhibition. With lower concentrations of ATP the amount of gas evolved is too small for accurate measurement. Higher concentrations of ATP introduce manometric errors, since their buffering effect results in bicarbonate retention.

During these experiments it was observed that different hexokinase preparations varied greatly in their sensitivity to DL-glyceraldehyde. The stable dialyzed and lyophilized extract from acetone-dehydrated beef brain was strongly inhibited by glyceraldehyde. Extracts prepared in the same manner but which had not been centrifuged to rid them of insoluble particles were less sensitive. If the brain tissue was frozen before the enzyme extract was prepared, the hexokinase activity seemed very little inhibited by glyceraldehyde.³ Hexokinase from liver tumors of rats likewise was inhibited only slightly. Yeast hexokinase was not at all inhibited by glyceraldehyde. These data will not be tabulated here, since similar results will be presented with other data in a later section. Glyceraldehyde *per se* seemed not to be the inhibitor of hexokinase, since it had almost no effect on certain preparations of this enzyme.

To obtain further information about the nature of the reactions involved in the inhibition of hexokinase, phosphate partition was studied in control experiments and in those in which DL-glyceraldehyde had caused partial inhibition.

The results which contributed to the solution of the present problem are summarized in Table I. In the control flasks appreciable quantities of hexose phosphate and of triose phosphate accumulated. Further breakdown of the triose phosphate is inhibited by iodoacetate. In the vessels containing sufficient DL-glyceraldehyde to inhibit hexokinase about 60 per

³ In a typical experiment fructose phosphorylation by the lyophilized and dialyzed preparation from fresh brain was inhibited nearly 100 per cent by 1.6×10^{-4} M DL-glyceraldehyde. The same concentration of glyceraldehyde inhibited a similar preparation from frozen brain only 20 per cent.

cent, some hexose phosphates accumulated but no triose phosphate could be detected. Pyruvate, which relieves glyceraldehyde inhibition of glycolysis in some tissues (2) but not in brain, had no effect on the distribution of phosphoric esters. Apparently, brain tissue aldolase had condensed ketotriose phosphate with glyceraldehyde just as does muscle aldolase (25). Meyerhof, Lohmann, and Schuster (25) discovered that the enzyme which reversibly cleaves HDP to 1 mole of dihydroxyacetone phosphate and 1 of D-glyceraldehyde-3-phosphate could condense a variety of aldehydes with dihydroxyacetone phosphate. They found that when D-glyceraldehyde was the condensing aldehyde D-fructose-1-phosphate was produced. When racemic glyceraldehyde was used, an equimolar mixture of D-fructose-1-phosphate and presumably L-sorbose-1-phosphate was formed. Since the equilibrium for the enzymatic condensation reaction is far toward the side of ketose-1-phosphate formation (25), the

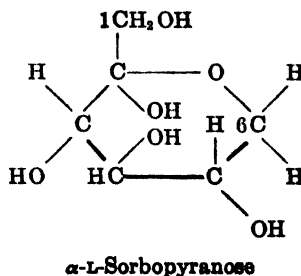
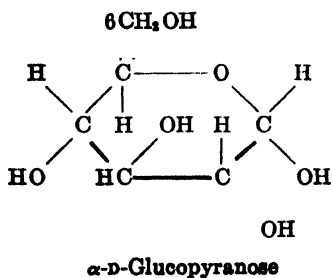
TABLE I

Effect of Glyceraldehyde on Phosphate Distribution

Phosphorus values for "hexose phosphate" were determined by difference; *i.e.*, acid-soluble organic phosphorus minus that present in triose phosphate and the adenine nucleotides. Glucose, 0.01 M; ATP, 0.003 M.

Conditions	Hexose phosphate formed	Triose phosphate formed
	$\mu\text{M P}$	$\mu\text{M P}$
Blank (no glucose).....	0	0
Control.....	4.8	2.9
+ 3.7×10^{-3} M DL-glyceraldehyde..	3.2	0
+ 3.7×10^{-3} " " and		
3.3×10^{-3} M pyruvate.....	3.6	0

virtually complete removal of triose phosphate (Table I) is to be expected. The question then arose whether these ketose-1-phosphates might influence the hexokinase reaction. D-Fructose-1-phosphate is a naturally occurring ester (43-45) and has been shown by Cori and Slein (42) to be phosphorylated by muscle phosphohexokinase to HDP just as is fructose-



6-phosphate. It seemed possible, however, that L-sorbose-1-phosphate might be an inhibitor because of the structural relationship between D-glucose and L-sorbose.

An enzymatic synthesis (Equations 1 to 3) of the two ketohexose-1-phosphates in question was carried out with D- and L-glyceraldehyde, respectively, in the presence of purified rabbit muscle aldolase (Table II). Hexose diphosphate was used as a source of triose phosphate. After deproteinization, aliquots of the filtrates were analyzed for triose phosphate. The remainders were fractionated according to LePage (34) and the barium-soluble, alcohol-insoluble fractions which contained hexose

TABLE II

Synthesis of Hexokinase Inhibitor from Hexose Diphosphate and L-Glyceraldehyde by Crystalline Rabbit Muscle Aldolase

Each tube contained 5 μ M of K-HDP and 100 γ of crystalline aldolase (from rabbit muscle by the procedure of Taylor *et al.* (46)); not recrystallized. The crystals had been collected by filtration, dried *in vacuo*, and stored at 5° for 2 months before use). Temperature 25°; Tube 1 incubated 15 minutes; Tubes 2, 3, and 4 incubated 30 minutes.

Tube No.	Glyceraldehyde added	Triose phosphate formed	Hexokinase activity*
	μ M	μ M	per cent
1	0	2.2	107
2	0	3.3	104
3	L isomer, 130	1.6	15
4	D " 130	0.7	96
			19†

* Hexokinase activity in the presence of one-tenth of the hexose monophosphate fraction from the above filtrate. Control with glucose as substrate = 100.

† Hexokinase activity in the presence of 0.5 μ M of synthetic L-sorbose-1-phosphate. The hexose monophosphate fraction assayed from Tube 3 contained 0.68 microatom of organically bound phosphorus

monophosphates were separated, washed thoroughly to free them of traces of glyceraldehyde, and dried. The precipitates were taken up in water and freed of barium with sulfate, and aliquots of these solutions were tested for possible inhibitory effects on the hexokinase system. Preliminary experiments (with smaller amounts of the HMP fraction) indicated that the fraction from Tube 3 inhibited glucose phosphorylation. The

- $$\begin{aligned} & \text{aldolase} \\ (1) \text{ Fructose-1,6-diphosphate} & \rightleftharpoons \text{dihydroxyacetone phosphate} + \text{D-glyceraldehyde phosphate} \\ (2) \text{ Dihydroxyacetone phosphate} & + \text{D-glyceraldehyde} \rightarrow \text{D-fructose-1-phosphate} \\ (3) \text{ Dihydroxyacetone phosphate} & + \text{L-glyceraldehyde} \rightarrow \text{L-sorbose-1-phosphate} \end{aligned}$$

suspected ester in this tube was sorbose-1-phosphate, and, since this was being prepared in connection with another study (39), it was tested in the final experiment shown in Table II. The results show that no inhibitor was produced in the tubes which contained only HDP or HDP plus D-glyceraldehyde.⁴ The hexose monophosphate fraction from Tube 3, in which L-glyceraldehyde had been incubated with HDP and aldolase, strongly inhibited hexokinase. Furthermore, synthetic L-sorbose-1-phosphate inhibited hexokinase to a similar degree. A comparison was then made of the effect of synthetic L-sorbose-1-phosphate on the hexokinases of different sources which varied so greatly in their sensitivity to glyceraldehyde. The results in Table III show that L-sorbose-1-phosphate was equally inhibitory to preparations which were strongly inhibited by glyceraldehyde and to those which were less sensitive to the free triose. These

TABLE III

Susceptibility of Hexokinase to Glyceraldehyde Inhibition

All enzyme preparations were lyophilized and dialyzed. Experimental conditions as described under "Methods and materials." Inhibition calculated from the 5 to 35 minute period after tipping ATP from the side arms into the reaction mixture. Note that the concentration of L-sorbose-1-phosphate is only one-twentieth that of DL-glyceraldehyde.

Enzyme source	Inhibition by		
	1.6×10^{-3} M DL-glyceraldehyde	8×10^{-5} M L-sorbose-1-phosphate	3×10^{-3} M L-sorbose
	per cent	per cent	per cent
Fresh brain	100	60	0
Frozen "	20	59	0
Liver tumor	12	63	0

results indicated that L-glyceraldehyde *per se* is probably not an inhibitor of hexokinase and that the actual inhibitor is L-sorbose-1-phosphate which is formed by Reaction 3 above.

Neither DL-glyceraldehyde (0.006 M) nor L-sorbose-1-phosphate (0.003 M) inhibited partially purified yeast hexokinase.

In Table IV are summarized experiments to test the specificity and the nature of the inhibitory effect of L-sorbose-1-phosphate. This ester inhibited the phosphorylation of glucose, fructose (Experiment A), and mannose (other experiments). It did not inhibit the conversion of glu-

⁴ Since the HMP from Tube 4 (presumably D-fructose-1-phosphate) did not increase the rate of CO₂ evolution, it apparently was not phosphorylated by brain phosphohexokinase. This was confirmed in direct tests with enzymatically prepared fructose-1-phosphate and is in agreement with the recent report of Slein (47).

cose-6-phosphate or fructose-6-phosphate to fructose diphosphate nor did it affect the phosphorylation of tagatose-6-phosphate (48) (Experiment C). In its specificity the ester parallels L-glyceraldehyde (Fig. 2). L-Sor-

TABLE IV

Effect of Varying Concentrations of Reactants and Inhibitors on Hexokinase and Phosphohexokinase Activity

In all experiments except the one noted activity is calculated from the 5 to 35 minute period after tipping in ATP. Each inhibition value is calculated by comparison with the activity in a control flask containing no inhibitor but all other additions as indicated.

Experiment	Inhibitor		Substrate		ATP	Inhibition
		<i>M</i>		<i>M</i>	<i>M</i>	<i>per cent</i>
A	Sorbose-1-PO ₄	8×10^{-5}	Glucose	0.005	0.004	59
	"	1.6×10^{-4}	"	0.005	0.004	82
	"	5.0×10^{-4}	"	0.005	0.004	91-100
	"	1.6×10^{-4}	Fructose	0.005	0.004	55
	"	5.0×10^{-4}	"	0.005	0.004	83
B	"	8×10^{-5}	Glucose	0.001	0.004	55 (80)*
	"	8×10^{-5}	"	0.002	0.004	62
	"	8×10^{-5}	"	0.005	0.004	60
	"	8×10^{-5}	"	0.035	0.004	67
	"	8×10^{-5}	Fructose	0.001	0.004	23
	"	8×10^{-5}	"	0.002	0.004	25
	"	8×10^{-5}	"	0.005	0.004	48
C	"	8×10^{-4}	Glucose-6-PO ₄	0.006	0.004	0
	"	8×10^{-4}	Fructose-6-PO ₄	0.006	0.004	0
	"	8×10^{-4}	Tagatose-6-PO ₄	0.006	0.004	0
	"	8×10^{-5}	Glucose	0.002	0.004	55
	Sorbose-6-PO ₄	1.6×10^{-3}	Glucose-6-PO ₄	0.006	0.004	0
	"	1.6×10^{-3}	Fructose-6-PO ₄	0.006	0.004	0
	"	1.6×10^{-3}	Tagatose-6-PO ₄	0.006	0.004	0
D	"	1.6×10^{-3}	Glucose	0.002	0.004	0
	Sorbose-1-phosphate	8×10^{-5}	"	0.002	0.002	58
	"	8×10^{-5}	"	0.002	0.004	55
	"	8×10^{-5}	"	0.002	0.008	58
E	"	8×10^{-5}	Fructose	0.01	0.002	35
	"	8×10^{-5}	"	0.01	0.004	40
	"	8×10^{-5}	"	0.01	0.008	39

* Value for first 20 minutes before glucose became limiting in control flask.

bosc-6-phosphate (39) (Experiment C) and free L-sorbose (Table III) are not inhibitory. In most experiments L-sorbose-1-phosphate inhibited the phosphorylation of glucose more than that of fructose (Table IV, Experiments A and B). However, in one experiment fructose phosphorylation

was more strongly inhibited. No evidence was obtained that hexoses (Experiment B) or ATP (Experiments D and E) could competitively reverse the inhibitory effect of L-sorbose-1-phosphate.

DISCUSSION

The structural similarity between L-sorbose-1-phosphate and the product of the hexokinase reaction, namely glucose-6-phosphate, is striking.

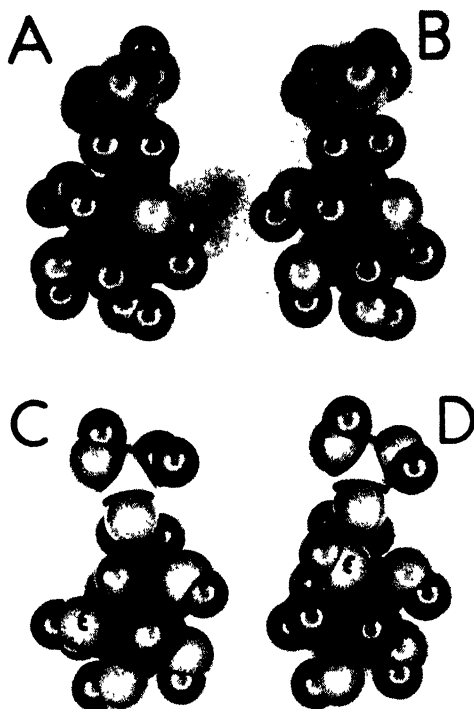


FIG. 3. *A* and *C*, α -D-glucopyranose-6-phosphate, *B* and *D*, α -L-sorbofuranose-1-phosphate.

In Fig. 3 are shown Hirschfelder models of α -D-glucopyranose-6-phosphate and of α -L-sorbofuranose-1-phosphate in their most probable (49, 50) conformation, one of two possible "chair" forms. Viewed from one side, the 2 molecules are indistinguishable (*A* and *B*). On the opposite surface (*C* and *D*) the only difference apparent is a transposition of one hydroxyl group and 1 hydrogen atom. In the glucose molecule the polar (ϵ in Hassel's nomenclature) hydroxyl is that on carbon atom 1, while in the sorbose molecule it is attached to the ring-forming carbon 2. As will be

pointed out elsewhere, variations in configuration at these sites do occur in substrates which are phosphorylated by hexokinase of beef brain. This strongly suggests that the inhibitor combines with that portion of hexokinase which has affinity for glucose and thus prevents the substrate from combining. Yet no competitive relationship between glucose or fructose and L-sorbose-1-phosphate has been established (Table IV). Experiments in which varying concentrations of hexokinase and several levels of L-sorbose-1-phosphate were employed gave evidence (51) for irreversible combination of the enzyme with the inhibitor. A detailed study of the mechanism by which L-sorbose-1-phosphate inhibits animal hexokinase must await further purification of this enzyme, since phosphatases and other protein contaminants interfere with kinetic studies.

The mechanism by which pyruvate reverses inhibition of glycolysis by glyceraldehyde has not been studied in the present investigation. However, one might postulate that by keeping coenzyme I in its oxidized form added pyruvate might increase the rate of triose phosphate oxidation, leaving less to condense with L-glyceraldehyde to form the inhibitor. Competition, between D- and L-glyceraldehyde, for triose phosphate might also explain why L-glyceraldehyde in the racemate was less inhibitory than when tested in optically active form (Fig. 1).

SUMMARY

The hexokinase activity of a dialyzed and lyophilized extract of acetone-dried beef brain was strongly depressed when L-glyceraldehyde was added. D-Glyceraldehyde was not inhibitory. Additions of L-glyceraldehyde did not inhibit phosphohexokinase.

Since different preparations of hexokinase varied in their sensitivity to L-glyceraldehyde, the inhibitory action of the latter appeared to be an indirect one.

Evidence was obtained that the hexokinase inhibitor was formed when L-glyceraldehyde condensed with triose phosphate to form L-sorbose-1-phosphate.

Synthetic L-sorbose-1-phosphate strongly inhibited brain and tumor hexokinase but not phosphohexokinase.

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PHOSPHORIC ESTERS OF BIOLOGICAL IMPORTANCE

V. THE SYNTHESIS OF L-SORBOSE-1-PHOSPHATE AND L-SORBOSE-6-PHOSPHATE*

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Interest in the phosphoric esters of sorbose arose from the observation of Embden, Schmitz, and Wittenberg in 1913 (1) that perfusion of liver with D-glyceraldehyde gave rise to both D-glucose and L-sorbose (at that time called *d*-sorbose). Later Meyerhof, Lohmann, and Schuster (2) found that in the presence of muscle aldolase D-glyceraldehyde condensed with dihydroxyacetone phosphate to yield fructose-1-phosphate. The racemic aldehyde in turn gave rise to an equimolar mixture of D fructose-1-phosphate and L-sorbose-1-phosphate. The action of phosphatases on the latter to yield free sorbose is a plausible explanation of the findings of Embden *et al.*

This condensation of the L component of synthetic glyceraldehyde to produce sorbose derivatives appeared to be mainly of theoretical interest. However, some time ago preliminary evidence was obtained¹ which indicated the possible natural occurrence of phosphoric esters of a triose of L configuration. It has since been found that, although partially purified preparations of aldolase and isomerase would not condense L-glyceraldehyde-3-phosphate with dihydroxyacetone phosphate (foot-note, p. 349 (3)), crude preparations did catalyze the condensation of the L ester. These results will be reported in a subsequent publication.

In view of the findings discussed above it seemed possible that phosphoric esters of L-sorbose might occur in tissues and the synthesis of such esters was therefore undertaken. During the course of this work it was found in a separate investigation (4) that a substance formed from L-glyceraldehyde, presumably L-sorbose-1-phosphate, is a powerful inhibitor of hexokinase. Synthetic L-sorbose-1-phosphate proved to be strongly inhibitory to this enzyme, thus confirming the identity of the enzymatically produced inhibitor.

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¹ Unpublished results from this laboratory, 1944.

A convenient starting material for the synthesis of sorbose-1-phosphate is the 2,3:4,6-diisopropylidene sorbose of Reichstein and Grüssner (5). The free hydroxyl on carbon atom 1 is esterified by treating with diphenyl chlorophosphonate (6). The phenyl groups are removed from the resulting ester by hydrogenation and after hydrolytic removal of the isopropylidene groups the phosphate ester is isolated as the barium salt.

A second and less satisfactory synthesis involves the phosphorylation of a crude dibenzylidenesorbose sirup, also prepared by Reichstein and Grüssner (5). These workers oxidized the crude mixture with potassium permanganate and obtained an acid, $C_{20}H_{18}O_7$, indicating that the original mixture contained a dibenzylidenesorbose with a free primary hydroxyl group. By esterifying the crude material with either *p*-toluenesulfonyl chloride or benzoyl chloride, it has been possible to obtain crystalline compounds. After tedious purification these derivatives could be hydrogenated over palladium on charcoal (7) to yield the well characterized 1-tosyl (8, 9) and 1-benzoyl-L-sorbose (8). Platinum, on the other hand, only led to partial reduction of the aromatic nuclei. The inert nature of the tosyl group in 1-tosyl-2,3:4,6-diisopropylidene-L-sorbose toward the sodium iodide exchange reaction (10) was also evident in the case of tosylated dibenzylidenesorbose. From the present work it may be inferred that at least one component of the benzylidinated sorbose sirup is a dibenzylidenesorbose with a free hydroxyl group on carbon atom 1.

This open hydroxyl was then esterified with diphenyl chlorophosphonate to yield a crystalline product. Attempted hydrogenolysis of the benzylidene residues with palladium led largely to the formation of inorganic phosphate. With platinum, the hydrogen absorbed was almost sufficient to account for the complete removal of the phenyl and benzylidene groups. Although the barium salt isolated from this reaction inhibited hexokinase as does L-sorbose-1-phosphate (4), it was low in phosphorus and probably contaminated with partially reduced compound.

It has been reported by Cori and Slein (11) that a phosphohexokinase fraction of rabbit muscle will phosphorylate fructose-1-phosphate to form fructose-1,6-diphosphate. Considering the structural similarity of the fructose monophosphates and L-sorbose-6-phosphate about carbon atoms 3 and 4, it seemed of interest to prepare the latter sorbose ester and to determine whether it inhibited phosphohexokinase activity.

L-Sorbose-6-phosphate was synthesized by phosphorylation of 2,3-isopropylidene-L-sorbose. Previously it had been shown by Müller and Reichstein (10) that treatment of the latter compound with *p*-toluenesulfonyl chloride yielded the 1,6-ditosyl derivative. By analogy it may be assumed that diphenyl chlorophosphonate would yield the corresponding 1,6-diester. If the phenyl groups are reductively cleaved in the usual manner, the free acid formed may be used to hydrolyze the isopropylidene

residues. Assuming that L-sorbose-1,6-diphosphate behaves like the fructose diester (12), then the more labile 1-phosphate is also removed by this treatment. The relative insolubility of the tetrabasic barium salt of sorbose-1,6-diphosphate (*cf.* fructose diphosphate (12)) and barium phosphate facilitates their removal.² Upon the addition of acetone to the filtrate, the barium salt of L-sorbose-6-phosphate is obtained.

Synthetic L-sorbose-6-phosphate did not inhibit the phosphorylation of glucose-6-phosphate (13), fructose-6-phosphate (12), or tagatose-6-phosphate (14) by a dialyzed and lyophilized extract of beef brain. Since it likewise did not inhibit the phosphorylation of glucose or fructose, even when added in comparatively high concentration, it was considered to be free of L-sorbose-1-phosphate.

EXPERIMENTAL³

2,3:4,6-Diisopropylidene-L-sorbose-1-diphenyl Phosphate—To 5 gm. of 2,3:4,6-diisopropylidene-L-sorbose⁴ dissolved in cold (0°) dry pyridine, 7.25 gm. of diphenyl chlorophosphonate were added dropwise with stirring. Near the end of the addition white crystals of pyridine hydrochloride appeared. The reaction mixture was left overnight at 4° and then decomposed by pouring onto 500 ml. of ice and water. The oil which settled out refused to crystallize and therefore was extracted with three 100 ml. portions of chloroform. The latter solution was then washed successively with ice-cold solutions of dilute hydrochloric acid and sodium bicarbonate. Following a final water wash the chloroform solution was dried with anhydrous sodium sulfate and the solvent completely removed under a vacuum, leaving a viscous residue, 7.8 gm. (83 per cent of theory).

$[\alpha]_D^{25} = -11.7^\circ$ ($c = 5.0$ in chloroform)			
$C_{24}H_{30}O_8P$ (492.4). Calculated. C 58.52, H 5.93, P 6.29]			
Found.	" 58.47,	" 5.81,	" 6.36
	" 58.27,	" 5.97,	" 6.45

Barium L-Sorbose-1-phosphate—5 gm. of 2,3:4,6-diisopropylidene-L-sorbose-1-diphenyl phosphate were dissolved in 25 ml. of redistilled methanol together with 1 ml. of water and reduced in the presence of 0.5 gm. of platinum oxide (17). After 6 hours the reduction stopped when 9.5 moles of hydrogen had been consumed, indicating partial removal and hydrogenation of the isopropylidene residues. The catalyst was removed

² Partially purified sorbose diphosphate has been prepared from this mixed precipitate.

³ Phosphorus was determined by the method of King (15). Barium was determined by the method of Frediani and Babler (16), 1939, adapted for the Evelyn colorimeter. Carbon and hydrogen analyses were performed by Clark Microanalytical Laboratory, Urbana, Illinois, except one. All melting points are corrected.

⁴ Generously supplied by Dr. Wm. Sullivan, Hoffmann-La Roche, Inc., Nutley, New Jersey.

by filtration and the solvents by distillation (bath temperature 50°) under a vacuum (10 mm.). Then the residual oil was taken up in 20 ml. of water and heated on a steam bath for 35 minutes to hydrolyze the remaining isopropylidene groups. Following neutralization of the solution to the pink color of phenolphthalein with saturated barium hydroxide, the inorganic phosphate was separated by centrifugation or filtration through a diatomaceous earth pad (Johns-Manville, Celite). Upon the addition of 2 volumes of cold acetone the barium salt of L-sorbose-1-phosphate precipitated. Purification was achieved by dissolving the salt in 50 ml. of water, removing the insoluble material, and reprecipitating with acetone. The barium salt was collected by centrifugation and washed several times with acetone and then with peroxide-free ether. By drying in a vacuum desiccator over sulfuric acid a powdery white amorphous solid was obtained; yield, 1.5 gm.

$[\alpha]_D^{25} = -7.2^\circ$ ($c = 2.5$ in 0.104 N HCl)
 $C_6H_{11}O_5PBa \cdot 2H_2O$ (431.6). Calculated. C 16.69, H 3.50, P 7.19, Ba 31.91
 Found. " 16.83, " 3.62, " 7.31, " 31.5

Hydrolysis of Barium L-Sorbose-1-phosphate in 1 N HCl at 98.5°

Time, min.	5	10	20	30	60
Hydrolysis, %	35.8	58.9	83.4	90.8	95.0
k , min. ⁻¹	0.089	0.089	0.090	0.080	

Crystalline Potassium Hydrogen L-Sorbose-1-phosphate—The barium salt of L-sorbose-1-phosphate (1.5 gm.) was passed slowly through a repeatedly acid- and base-washed column of Amberlite IR-100 in the potassium cycle. After evaporation of the barium-free effluent to 15 ml. under a vacuum (bath temperature, 40–50°), the solution was adjusted to pH 3 with glacial acetic acid. Ethanol was then added until a faint turbidity persisted, and the solution was allowed to stand at room temperature until crystals formed. Small periodic additions of alcohol were made until no more crystals separated. They were collected on a sintered glass funnel, washed with absolute ethanol and then with ether, and dried *in vacuo* over sulfuric acid; yield, 0.55 gm. Once dried in this manner the compound appeared quite stable, and, since it is readily soluble in water, is most convenient for study in biological systems.

$[\alpha]_D^{25} = -16.5^\circ$ ($c = 2.0$ in water)
 $C_6H_{12}O_5PK$ (298.3). Calculated. C 24.15, H 4.06, P 10.40, K 13.11
 Found.⁵ " 24.01, " 4.20, " 10.3, " 13.3

⁵ Carbon and hydrogen analyses performed by Micro-Tech Laboratories, Skokie, Illinois. We wish to thank Mr. R. R. Grunert for the potassium analyses.

Hydrolysis of Potassium Hydrogen L-Sorbose-1-phosphate in 1 N HCl at 98.5°

Time, min.	5	10	15	20	30	65
Hydrolysis, %	37.2	61.1	74.7	83.1	92.1	94.8
<i>k.</i> , min. ⁻¹	0.092	0.094	0.092	0.089	0.080	

Dibenzylidene-L-sorbose—The condensation of L-sorbose and benzaldehyde was catalyzed with dry hydrogen chloride by the method of Reichstein and Grüssner (5). Following removal of the hydrogen chloride and unused L-sorbose, the only deviation from their procedure was the extraction of the excess benzaldehyde with low boiling petroleum ether. The last traces of the solvent were then readily distilled under a vacuum.

1-Tosyl Dibenzylidene-L-sorbose—17 gm. of dibenzylidene-L-sorbose were dissolved in 50 ml. of cold (0°) dry pyridine and to this solution were added 10 gm. of pulverized *p*-toluenesulfonyl chloride in one batch. The insoluble pyridine hydrochloride rapidly separated from the reaction mixture which was then left at 4° for 24 hours. Pouring the brown mixture onto 400 gm. of ice and water caused the separation of an oily yellow precipitate. Solidification could be induced by replacing the aqueous phase with a small amount of 95 per cent ethanol. The precipitate was removed by filtration; yield 12.4 gm., 51.1 per cent, m.p. 124–144°. Much of the color was removed by treating in ethanolic solution with charcoal (Nuchar G.F.O.), and several recrystallizations from absolute ethanol raised the melting point to 160.5–162.0°.

$[\alpha]_D^{25} = -10.6^\circ$ ($c = 5.0$ in chloroform)
 C₂₇H₂₈O₈S (510.5). Calculated. C 63.52, H 5.13, S 6.28
 Found. " 63.40, " 5.22, " 6.19

Reduction of Tosyl Dibenzylidene-L-sorbose

Platinum Reduction—The reduction of tosylated dibenzylidene-L-sorbose was carried out in glacial acetic acid with Adams' platinum oxide catalyst (17). Under these conditions a variable amount of hydrogen (from 5.0 to 8.2 moles per mole of tosyl dibenzylidene-L-sorbose) was consumed. This indicated that the benzylidene rings were being reduced as in the case of 1,6-dibenzoylbenzylidene-fructose studied by Brigl and Müller (6). In general, the products fell into two classes, a low melting compound (m.p. 110°), and a high melting compound (m.p. 142°). There was, however, no obvious correlation between the melting point and the quantity of hydrogen consumed.

Palladium Reduction—5 per cent palladous chloride on charcoal was prepared according to the method of Mozingo (7). Following reduction of the catalyst in glacial acetic acid the sample of tosylated dibenzylidene-L-sorbose to be reduced was added in a solution of the same solvent. In

the presence of dry hydrogen the reduction proceeded rapidly and in three runs 4.7, 4.8, and 5.1 moles of hydrogen were absorbed respectively. The catalyst was removed by filtration and the filtrate evaporated to an oil under a vacuum. This residue was then taken up in absolute ethanol and low boiling petroleum ether was added until turbidity persisted. Upon standing overnight in the refrigerator fine white crystals separated; m.p. 113–118°. Recrystallization from the same solvent pair gave a product which melted to a red residue; m.p. 121–122°, $[\alpha]_D^{25} -11.0^\circ$, ($c = 4.0$ in pyridine). Admixture with authentic 1-tosyl-L-sorbose (8) failed to lower the melting point.

Sodium Iodide Exchange—0.5 gm. of each of the substances noted below was dissolved in 15 ml. of methyl isobutyl ketone and refluxed in the presence of 0.5 gm. of sodium iodide with the result shown: 1-tosyl-L-sorbose, immediate precipitate; 1-tosyl-2,3:4,6-diisopropylidene-L-sorbose, no reaction; 1,6-ditosyl-2,3-isopropylidene-L-sorbose, slow precipitation; 1-tosyl dibenzylidene-L-sorbose, no reaction.

1-Benzoyl Dibenzylidene-L-sorbose—15 gm. of dibenzylidene-L-sorbose sirup were dissolved in 75 ml. of dry pyridine and cooled to 0°. This solution was then stirred vigorously and 7.3 gm. of benzoyl chloride added under anhydrous conditions. After 36 hours at 4° the reaction mixture was poured onto ice and water and the product extracted with three 100 ml. portions of chloroform. The latter solution was freed of pyridine with ice-cold dilute hydrochloric acid and of the acid with ice-cold bicarbonate solution. It was then dried with anhydrous sodium sulfate and the solvent distilled under a vacuum. The residual oil was refluxed under low boiling petroleum ether and a light brown solid separated; m.p. 73–85°, yield 11.5 gm. Purification was attained only by crystallization through the following sequence: absolute ethanol (m.p. 120–122°), benzene plus low boiling petroleum ether (m.p. 130–132°), acetone plus water (m.p. 140–141°), and absolute ethanol (m.p. 140–140.5°).

$[\alpha]_D^{25} = -2.2^\circ$ ($c = 5.0$ in chloroform)	
$C_{27}H_{34}O_7$ (460.5).	Calculated. C 70.41, H 5.25
	Found. " 70.69, " 5.30
	" 70.41, " 5.05

Reduction of 1-Benzoyl Dibenzylidene-L-sorbose

Platinum Reduction—2 gm. of 1-benzoyl dibenzylidene-L-sorbose were dissolved in 25 ml. of glacial acetic acid and reduced with hydrogen at atmospheric pressure in the presence of 0.2 gm. of Adams' platinum oxide catalyst (17). When 10 moles of hydrogen per mole of compound had been consumed (4 hours) the reaction stopped. The catalyst was removed by filtration and the filtrate taken to dryness under a vacuum. By repeatedly dissolving the residue in absolute ethanol and evaporating

at reduced pressure all acetic acid was removed. Upon scratching a chilled ethanolic solution, fine white crystals, m.p. 94–95.5°, were deposited. Considering this melting point and the fact that this material was very soluble in both ether and low boiling petroleum ether, it was evident that it could not be 1-benzoyl-L-sorbose. Rather it must arise by partial hydrogenation of the benzylidene groups without cleavage from the sugar as noted by Brigl and Müller (6).

Palladium Reduction—Preliminary hydrogenations of 1-benzoyl dibenzylidene-L-sorbose were carried out in the presence of the hydrogen chloride developed by reduction of palladous chloride (7). The product of the reaction was a small amount of high melting compound (m.p. 186–189°).

Subsequently the palladium on charcoal was thoroughly washed with glacial acetic acid, following reduction of the catalyst. The benzoylated compound, dissolved in glacial acetic acid, was then added and hydrogen admitted to the system. Sufficient hydrogen (4.2 moles) was consumed for hydrogenolysis of the benzylidene residues. Following removal of the solvent, the residual oil was taken up in absolute ethanol and crystallized by the addition of low boiling petroleum ether. Recrystallization from the same solvent pair yielded 0.5 gm. of a compound melting at 132–133.5°, $[\alpha]_D^{25} -4.8^\circ$, ($c = 5.0$ in methanol). Sullivan (8) reported a melting point of 133.5° and a rotation of $[\alpha]_D^{20} -3.4^\circ$, ($c = 5.0$ in methanol).

Dibenzylidene-L-sorbose-1-diphenyl Phosphate—10 gm. of L-sorbose⁴ were condensed with 50 ml. of freshly distilled benzaldehyde in the presence of 1.3 gm. of dry hydrogen chloride, and 18.5 gm. of dibenzylidene-L-sorbose were isolated. This sirup was dissolved in 50 ml. of dry pyridine, and cooled to 0°, and to the stirred solution 16.2 gm. of diphenyl chlorophosphonate were added slowly. The light brown reaction mixture rapidly filled with pyridine hydrochloride and was left overnight at 4°. It was decomposed by being poured slowly, with stirring, into a mixture of ice and water. Upon the addition of seed crystals, originally obtained by prolonged refluxing of the oily product freed of pyridine and water with low boiling petroleum ether, solidification proceeded rapidly; yield 13.6 gm. Purification was most easily achieved by adding water to a very dilute acetone solution of the solid. The pure product crystallized in a woolly mass, m.p. 135–136.5°.

$$[\alpha]_D^{25} = -22.4^\circ \quad (c = 5.0 \text{ in chloroform})$$

$C_{22}H_{19}O_5P$ (588.6). Calculated. C 65.23, H 4.97, P 5.27

Found. " 65.30, " 5.13, " 5.38

Reduction of Dibenzylidene-L-sorbose-1-diphenyl Phosphate

Palladium Reduction—Palladium on charcoal (7) in glacial acetic acid was used in an attempt to cleave reductively the benzylidene residues.

However, even when the catalyst had been washed free of the hydrogen chloride, dephosphorylation was the predominant reaction.

Platinum Reduction—2 gm. of dibenzylidene-L-sorbose-1-diphenyl phosphate were dissolved in 25 ml. of glacial acetic acid and the reduction catalyzed with 0.2 gm. of platinum oxide. The reaction stopped in 4 hours after 17 moles of hydrogen had been consumed. Following removal of both catalyst and solvent, barium hydroxide was added to the pink color of phenolphthalein, and the salt precipitated by the addition of an equal volume of ethanol. An acidic aqueous solution of this material was then extracted with ether to free it of the end-products of reduction. By adjusting the solution to pH 9 with ammonium hydroxide and adding 2 volumes of ethanol the barium salt was recovered. The precipitate was suspended in 75 ml. of water, filtered, and the salt recovered from the supernatant upon the addition of ethanol. After drying with ethanol and ether the product strongly inhibited hexokinase activity (4), indicating it to be largely L-sorbose-1-phosphate. Analysis, however, indicated incomplete removal of the protecting groups: C 28.8, H 4.51, P 6.01 per cent.

2,3-Isopropylidene-L-sorbose-1,6-bis-(diphenyl Phosphate)—5 gm. of 2,3-isopropylidene-L-sorbose⁴ (18), dissolved in 30 ml. of dry pyridine, were esterified by the dropwise addition with stirring of 12.9 gm. (2.1 moles) of diphenyl chlorophosphonate. As the last of the acid chloride was added a white precipitate of pyridine hydrochloride settled out. The reaction mixture was left overnight at 4° and then poured, in a thin stream, into a vigorously stirred mixture of ice and water. The resulting pale yellow oil gradually solidified and was removed by filtration. Washing by repeated suspension in water and decantation yielded a solid, 13.2 gm., m.p. 79–86°, which still contained traces of pyridine. Repeated crystallization from ethanol alone or ethanol and water raised the melting point to 100.5–101.5° yield, 8.5 gm.

$$[\alpha]_D^{25} = -4.2^\circ \quad (c = 5 \text{ in chloroform})$$

$C_{32}H_{24}O_{12}P_2$ (684.6). Calculated. C 57.88, H 5.00, P 9.05

Found. " 58.00, " 5.01, " 8.95

" 58.00, " 4.80, " 9.04

Barium L-Sorbose-6-phosphate—The reduction of the tetraphenyl ester described above (5.0 gm.) was carried out in 25 ml. of glacial acetic acid or methanol with 0.5 gm. of platinum oxide (17) to catalyze the reaction. After the theoretical amount of hydrogen for cleavage and hydrogenation of the phenyl groups had been consumed (104 per cent) the catalyst was removed and the solvent evaporated under a vacuum. By repeated solution of the oily residue in absolute ethanol and removal of the solvent at

50° and 8 mm. the last traces of acetic acid could be removed. Deaceto-
nation and hydrolytic removal of the 1-phosphate were achieved by dis-
solving the viscous material in 20 ml. of water and heating on the steam
bath for 45 minutes. On adjusting the solution to the pink color of phe-
nolphthalein with saturated barium hydroxide, a copious white precipitate
of the barium salts of sorbose diphosphate² and inorganic phosphate ap-
peared. Following removal of the precipitate by centrifugation, 2 vol-
umes of acetone were added to the supernatant solution. The barium
L-sorbose-6-phosphate was freed of all traces of the diphosphate by taking
the salt into solution with water, adjusting the pH to 10 with barium
hydroxide, removing the insoluble material, and reprecipitating with ac-
etone. The barium salt was dried in the same manner as the 1-phosphate.

$$[\alpha]_D^{25} = -12.0^\circ \quad (c = 2.0 \text{ in water})$$

$C_6H_{11}O_9P\text{Ba}$ (395.5). Calculated, C 18.23, H 2.80, P 7.83, Ba 34.7

$C_6H_{11}O_9P\text{Ba} \cdot H_2O$ (413.5). Calculated, C 17.43, H 3.17, P 7.49, Ba 33.2

Found. " 17.16, " 3.17, " 7.57, " 33.3

Hydrolysis of Barium L-Sorbose-6-phosphate in 1 N HCl at

Time, min.	5	20	45	120	180	360
Hydrolysis, %	5.3	20.6	39.6	73.4	85.5	96.2
k , min. ⁻¹	0.0116	0.0115	0.0112	0.0110	0.0107	0.00905

SUMMARY

L-Sorbose-1-phosphate was synthesized by two procedures and isolated
as the amorphous barium salt or as the crystalline potassium hydrogen
salt.

The starting material for the first method was the well known 2,3:4,6-
diisopropylidene-L-sorbose in which the hydroxyl on carbon atom 1 is
known to be free. The second procedure involved the phosphorylation
of dibenzylidene-L-sorbose. Since the structure of the latter is not known,
it was converted to two new compounds, 1-tosyl-dibenzylidene-L-sorbose
and 1-benzoyl-dibenzylidene-L-sorbose. These were converted respectively
to the previously characterized 1-tosyl and 1-benzoyl derivatives of L-
sorbose, indicating that the dibenzylidene sorbose possesses a free hydroxyl
on carbon atom 1. L-Sorbose-1-phosphate synthesized by either of the
procedures strongly inhibited hexokinase.

Barium L-sorbose-6-phosphate was prepared by reduction and hydrol-
ysis of 2,3-isopropylidene-L-sorbose-1,6-bis-(diphenyl phosphate) which
was synthesized from 2,3-isopropylidene-L-sorbose and diphenyl chloro-
phosphonate.

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ISOLATION OF A CRYSTALLINE ALBUMIN FROM MILK*

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The relationship between proteins from blood serum and milk has been the subject of considerable study, with some contradictory results (1-5). This is especially true of albumins. Application of electrophoretic techniques to the analysis of whey proteins indicated the presence of a small component, consisting of approximately 3 to 5 per cent of the total protein, with the electrophoretic mobility of a blood serum albumin. In view of the recent isolation, crystallization, and characterization of blood serum albumin (6), it was of interest to crystallize bovine whey albumin and compare its properties with those of crystalline serum albumin.

EXPERIMENTAL

15 gallons of skim milk (approximately 48 liters of whey) were treated by the procedures described for the separation of casein and isolation of crystalline β -lactoglobulin (7). Casein was removed by isoelectric precipitation at pH 4.7. The whey was then separated into two main fractions at ammonium sulfate concentrations of 2.3 and 3.3 M and pH 6.0. The 3.3 M salt fraction was dialyzed to free it of salt, and the crystals of β -lactoglobulin formed at pH 5.3 were removed by centrifugation. The mother liquor, which contained a complex mixture of proteins amounting to about 80 gm. in 7 to 10 liters of salt-free solution, was fractionated further at an alkaline pH.

Step 1, a. Fractionation with Ammonium Sulfate in Presence of Borate—The mother liquor was adjusted to pH 9.1 by the addition of sodium borate to a concentration of 0.05 M, and ammonium sulfate was added to a final concentration of 2.4 M. After the solution had stood at room temperature for a few hours, a sticky, brown-green, peroxidase-containing precipitate floated to the top and was removed (Precipitate 1, 17 gm.). The clear filtrate was brought to 2.6 M with ammonium sulfate, and the precipitate (Precipitate 2, 19 gm.) was removed by filtration. The protein remaining in the filtrate was precipitated completely with 3.4 M ammonium sulfate (Precipitate 3) at pH 5.0.

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Step 1, b—Precipitate 3 (28 gm.) was dissolved in water to a concentration of about 2 per cent, and separated into three fractions as in Step 1, *a*, but at concentrations of 2.6 (8 gm.), 2.8 (12 gm.), and 3.4 M ammonium sulfate.

Step 2. Alcohol Fractionation—The fraction precipitated with 3.4 M salt (7 gm.) was dissolved in a minimum amount of water and freed of the salt by dialysis. It was then equilibrated by dialysis with 10 per cent alcohol at pH 5.3 and ionic strength 0.005 to 0.01 at 3°. A clear, gelatinous precipitate formed that was removed by centrifugation. The alcohol concentration was then increased to 20 per cent, the precipitate removed by centrifugation, the temperature lowered to -5°, and the alcohol concentration increased to 33 per cent by the slow addition of 95 per cent alcohol to the supernatant. The precipitated protein obtained in this manner (5 gm.) consisted of 47 per cent albumin, as determined by electrophoretic analysis. The supernatant of the 33 per cent alcohol precipitate was adjusted to pH 4.8, ionic strength 0.02, and 40 per cent alcohol concentration at -5°. The precipitate formed was predominantly albumin mixed with three other globulin components (Fig. 1, *c*).

Step 3. Crystallization of Whey Albumin—The globulin components were separated, and the albumin was obtained in crystalline form by further fractionation with ammonium sulfate. The precipitate obtained with 40 per cent alcohol was dissolved in water and the alcohol removed by dialysis. The protein solution was made 0.1 M with dipotassium acid phosphate and 0.03 M with sodium caprylate. Ammonium sulfate was added to a concentration of 2.6 M; the precipitate that formed was removed by filtration, and a saturated ammonium sulfate solution was added until a slight turbidity appeared. After the solution had stood overnight at 3°, both crystals and some amorphous material formed. On warming to room temperature, the crystals dissolved. The amorphous material was removed by filtration, and, after the solution was cooled to 3°, feathery, needle-like crystals again formed (200 mg.). Increasing the salt concentration produced another crop of crystals.

Alternate Crystallization Procedures—The yield was increased considerably by isolating the albumin from the 33 per cent alcohol precipitate. This fraction (5 gm.) consisted of 47 per cent of albumin and 37 per cent of a component with an electrophoretic velocity of 4.4 sq. cm. volt⁻¹ sec.⁻¹ in veronal buffer at pH 8.4 (Fig. 2). After the alcohol was removed, the dissolved protein solution was made 0.1 M with respect to dipotassium acid phosphate and 0.02 M with sodium caprylate. It was then mixed with an equal volume of saturated ammonium sulfate. The precipitate that formed was essentially the protein with an electrophoretic mobility of 4.4 sq. cm. volt⁻¹ sec.⁻¹. The protein in the supernatant was completely precipitated

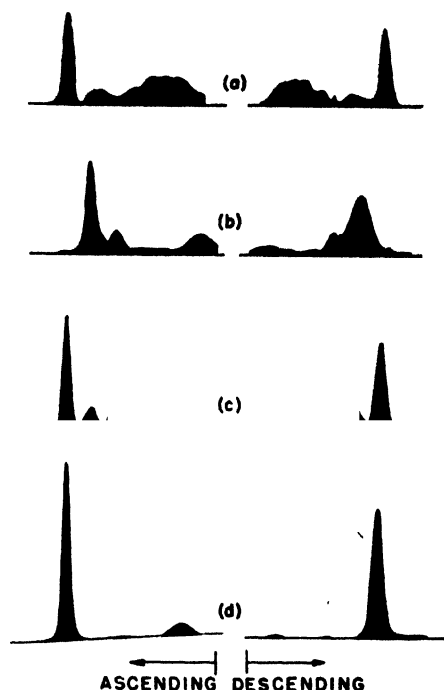


FIG. 1. Electrophoretic patterns in veronal buffer of 0.1 ionic strength (0.05 *N* sodium veronal, 0.05 *N* sodium chloride) at pH 8.4 of (a) bovine blood serum, (b) bovine milk whey, (c) whey protein precipitated with 40 per cent alcohol, and (d) crystalline bovine whey albumin after electrophoresis for 180 minutes.

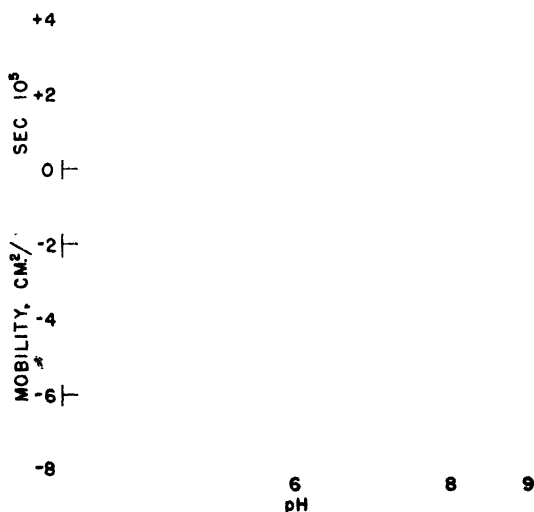


FIG. 2. Effect of pH on electrophoretic mobility of crystalline albumin of bovine whey in acetate and veronal buffers of 0.1 ionic strength.

with ammonium sulfate and treated as described in Step 3, yielding a crystalline albumin (500 mg.).

Step 1, *a* is modified by precipitating the 3.4 M ammonium sulfate fraction at pH 8.6. The filtrate is brought to pH 3 with 20 per cent trichloroacetic acid at 3°. The precipitate is allowed to settle overnight, the supernatant is decanted, and the remaining protein is removed by filtration. The paper containing the precipitate is macerated in dilute alkali solution, and the dissolved protein is fractionated further according to Step 3 to give a crystalline albumin.

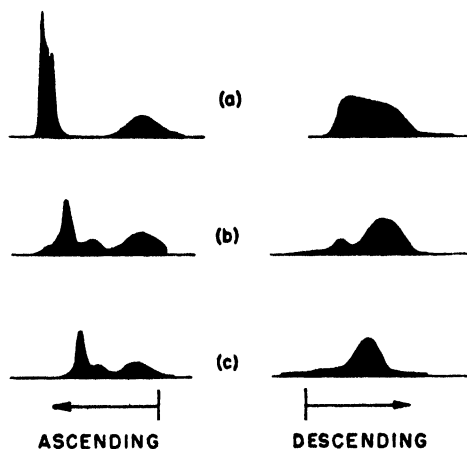


FIG. 3. Electrophoretic patterns of crystalline bovine serum albumin and whey albumin in acetate buffer at pH 4.0 after electrophoresis for 180 minutes. (a) Bovine serum albumin in buffer of 0.02 ionic strength, (b) crystalline whey albumin in buffer of 0.02 ionic strength, and (c) crystalline whey albumin in buffer of 0.1 ionic strength.

By these various procedures, from 0.5 to 1.0 gm. of crystalline whey albumin was obtained from 15 gallons of skim milk.

Properties

The crystalline whey albumin presented a single electrophoretic boundary in buffers of ionic strength 0.1 at pH values alkaline to the isoelectric point. In acetate buffers of ionic strength 0.1 or 0.02 at pH 4.0, the whey albumin showed a heterogeneous pattern similar to that described by Luetscher for serum albumin (8). The marked variations in components and mobility of crystalline bovine whey albumin as compared with serum albumin (Fig. 3) constituted the only electrophoretic differences observed between the two proteins. Mixtures of equal concentrations of the bovine whey and serum albumins migrated as a single component in veronal buffer at pH 8.4 and in acetate buffer at pH 4.8 with ionic strength 0.1.

The electrophoretic mobility of bovine serum albumin at 0° in veronal buffer of ionic strength 0.1 at pH 8.4 was -6.5×10^{-5} sq. cm. volt⁻¹ sec.⁻¹. Under similar conditions, the mobility of crystalline whey albumin was -6.7 . The electrophoretic mobility for serum albumin has been reported by Longworth and Jacobsen (9) as -6.64 in veronal buffer at pH 8.56 and by Cohn *et al.* (6) as -6.0 in veronal buffer at pH 8.6.

Other physicochemical constants for the crystalline bovine whey and serum albumins are listed in Table I. These show agreement within the limits of experimental error.

TABLE I
Comparison of Physicochemical Properties of Crystalline Bovine Serum and Milk Albumin

	Crystalline albumin	
	Serum*	Milk
Per cent nitrogen†	16.0	16.1
Mol. wt.‡	69,000	69,000
Optical rotation $[\alpha]_{488}^{25}$, degrees	-78 ± 2	-75
Isoelectric point	4.71	4.72
$E_{1\text{cm.}}^{1\%}$ at 280 mμ	6.6	6.6

* Constants for crystalline serum albumin were taken from the data reported by Cohn *et al.* (6) with the exception of the isoelectric point, which was reported by Longworth and Jacobsen (9). The constants for the milk albumin were determined in this laboratory.

† Ash and moisture-free basis. Determined by Dr. C. L. Ogg.

‡ Determined by Halwer *et al.* by a light-scattering technique (10).

§ Measured in acetate buffer of ionic strength 0.1 at pH 4.8.

|| The interpolated value for zero mobility obtained by electrophoresis of the albumin in acetate buffers of ionic strength 0.1.

Serological studies on the crystalline whey albumin by Dr. E. J. Coulson of the Allergen Division, United States Department of Agriculture, showed it to be identical with bovine serum albumin. These studies will be reported in detail by Dr. Coulson.

DISCUSSION

An electrophoretic study of the relation between the blood serum proteins and milk proteins in various stages of lactation has been reported by McMeekin *et al.* (11). The isolation of a crystalline albumin from milk with the properties of a crystalline serum albumin confirms and establishes the identity of the two proteins.

The use of small organic molecules as aids to crystallization with alcohol

has been amply discussed by Cohn *et al.* (6). The action, therefore, of caprylate as an adjunct for salt crystallization of bovine albumin is readily conceivable.

No explanation can be offered for the obvious difference in the electrophoretic patterns of the serum and whey albumins at pH 4.0. In the absence of specific experimental evidence, the argument that these variations reflect the interaction of the albumins with different anions or lipides can be considered only as a plausible and convenient hypothesis.

SUMMARY

It was possible to concentrate the albumin present in trace amounts in milk whey by salt and alcohol fractionation. Purified bovine milk albumin and serum albumin were crystallized with alkaline ammonium sulfate in the presence of phosphate and caprylate ions. Comparison of the physical, chemical, and serological properties of crystalline bovine milk albumin and serum albumin demonstrated the probable identity of these proteins.

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THE SEROLOGICAL RELATIONSHIP OF BOVINE WHEY ALBUMIN TO SERUM ALBUMIN

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Serological reactions are becoming increasingly important in the development of quantitative methods for the determination and characterization of native proteins. These reactions provide a basis for analytical methods that combine high sensitivity with a high degree of specificity. In contrast to conventional methods, which distinguish different proteins by their chemical and physical properties, serological methods reveal likeness or difference in structure.

In the preceding paper, Polis, Shmukler, and Custer (1) have described the isolation of a crystalline albumin present in trace amounts in the whey of cow's milk. They reported that this crystalline milk albumin exhibited physical and chemical properties attributable to crystalline bovine serum albumin. Our report describes the application of the Schultz-Dale (2, 3) technique to establish the identity of these two proteins.

EXPERIMENTAL

Materials—Crystalline preparations of milk albumin, serum albumin, and β -lactoglobulin were furnished by Dr. T. L. McMeekin. The bovine serum was obtained from the Bureau of Animal Industry, and the raw skim milk was supplied by the Bureau of Dairy Industry, United States Department of Agriculture.

Sensitization—In accord with previous reports (4), serum albumin proved to be a weak sensitizing antigen. Neither milk albumin nor serum albumin induced detectable antibody formation after intraperitoneal injection of a single 1 mg. dose in saline. Accordingly, the alum adjuvant (4, 5) was employed to enhance the antibody response to these proteins. Virgin female guinea pigs, weighing approximately 300 gm., were sensitized with subcutaneous injections of alum-precipitated protein. The sensitizing doses of the different preparations in terms of protein nitrogen were as follows: milk albumin 0.15 mg., bovine serum albumin 0.15 mg., β -lactoglobulin 0.15 mg., bovine serum 1.0 mg., and skim milk 3.0 mg. The larger amounts for the last two materials were used to provide adequate sensitizing doses of their minor components. The incubation period ranged from 40 to 90 days.

Schultz-Dale Technique—The Schultz-Dale experiments were conducted by the technique previously described (6). For qualitative tests each of the two uterine horns from a sensitized guinea pig was cut into two strips. One strip from each horn was stored in oxygenated Tyrode's solution. The other strips were mounted in the Dale baths. When the muscles were completely relaxed (30 to 45 minutes), the test dose was introduced and the response recorded on the kymograph. The baths were then flushed and refilled with fresh Tyrode's solution. When the muscle had again relaxed, an equal dose of the same antigen was added to insure desensitization before addition of a second preparation. If the strip failed to respond to the final test with antigen, the viability of the tissue was tested by addition of histamine. The two strips previously stored in Tyrode's solution were then used to repeat the same tests at a different dosage level or in a different sequence.

For quantitative tests (7), each uterus was cut into eight strips. Four strips were used to determine the minimal reactive dose of the purified protein to which the animal was sensitized, and the other four strips were used to determine the minimal reactive dose of the unknown mixture.

The anaphylactic response in excised smooth muscle is specific; only the particular protein that is used to sensitize will induce a response. Desensitization also is specific. If the animal is sensitized to two proteins and the uterus is desensitized to only one of them, the uterine tissue will still respond to the second protein.¹ However, relatively few purified proteins, including crystalline preparations, are serologically homogeneous (8). Hence, in the comparison of protein preparations from the same biological source by any serological method, one should be continually mindful of reactions caused by contaminants. Evidences of contamination may be revealed with the Schultz-Dale method by conducting tests at different dosage levels. Two homogeneous preparations of the same protein will induce like responses at all dosage levels. If one preparation is contaminated, they may still appear to be identical when tested at low levels, but will appear to be dissimilar at high levels. Conversely, two different proteins containing a common trace contaminant may cross-react only at high dosage levels.

Fortunately, the crystalline bovine serum albumin used in this study was one of the notably few proteins reported to be serologically homogeneous (8). Observations made in the present study indicated that the crystalline β -lactoglobulin also was free from other whey proteins.

¹ There are some exceptions to these rules. Thus some corresponding proteins from closely related biological sources give cross-reactions, but these are not quantitatively equivalent. Also, desensitization of a multiply sensitized uterus with a strong antigen will sometimes overwhelm subsequent reaction to a weaker antigen.

Results

The serological relationship of milk albumin and serum albumin was determined by the capacity of each preparation to induce reactions and by the specific desensitization to the other in uterine tissue from animals sensitized with milk albumin, bovine serum albumin, bovine serum, and skim milk.

In a qualitative experiment, a guinea pig was sensitized with serum albumin. 59 days later, two uterine strips from this animal were mounted in the Dale baths. Serum albumin induced a contraction in the first uterine strip and, after desensitization was established, the muscle did not respond to a subsequent test with milk albumin. In the second strip, milk albumin induced a reaction and desensitized the tissue to serum albumin. This experiment was conducted at doses of 1, 10, and 100 γ of protein nitrogen of the two preparations. All gave the same results. These responses showed that the preparations of milk albumin and serum albumin used contained a protein in common.

Similar experiments were conducted with uterine muscle from guinea pigs sensitized with crystalline milk albumin. In these tissues, milk albumin produced desensitization to serum albumin at all dosage levels. Likewise, when the tests were conducted at doses of 1 or 10 γ of protein N, serum albumin reacted and produced desensitization to milk albumin. However, when tests were made with doses of 100 γ of protein N, serum albumin did not completely give desensitization to milk albumin. These results implied that the two preparations contained a common protein and that the milk albumin contained, in addition, a protein not found in the serum albumin.

The comparative anaphylactogenic potencies of milk albumin and serum albumin were then determined by the quantitative method. In one experiment, eight uterine strips from an animal sensitized with serum albumin were used for quantitative comparison. The minimal reactive dose for both preparations was about 0.12 γ of protein N. This experiment was repeated with uterine muscle from eight other animals sensitized either with serum albumin or milk albumin. The minimal dose differed with different animals, but the antigenic potency of the two proteins was essentially the same in every case.

These results showed that the chief component of the milk albumin preparation was serologically identical with serum albumin and that the milk albumin contained a contaminant or contaminants capable of sensitizing the experimental animals. The contamination was not great enough to interfere in the Dale tests when small shock doses were employed, but was revealed by large test doses.

It was pertinent to identify one or more of the contaminants in the

milk albumin fraction before proceeding with experiments with blood serum or milk. The presence of β -lactoglobulin was suspected. Accordingly, Guinea Pig 1 was sensitized with milk albumin. The uterine muscles from this animal gave maximal responses with doses of β -lactoglobulin containing both 10 and 1 γ of protein N, but these reactions did not desensitize the muscle to subsequent addition of 1 γ of protein N from milk

TABLE I

Identification of β -Lactoglobulin in Milk Albumin and Demonstration of Second Contaminant

Additions of antigen to prove desensitization were always performed but are omitted from the tabular data.

Guinea pig No.	Sensitizing antigen	Uterine strip No.	Preparation added	Shock dose	Uterine contractions*
				<i>γ protein N</i>	
1	Milk albumin	1	β-Lactoglobulin	10	++++
			Milk albumin	1	++++
		2	β-Lactoglobulin	1	++++
			Milk albumin	1	++++
2	β-Lactoglobulin	1	“ “	10	0
		2	β-Lactoglobulin	1	++++
			Milk albumin	100	++
		3	β-Lactoglobulin	1	++++
			“ “	1	++++
			Milk albumin	100	0
		4	Histamine		++++
			Serum albumin	100	0
			β-Lactoglobulin	1	++++
		3	Milk albumin	1	“ “
Milk albumin	100				++
Serum albumin	100				0
2	Histamine				++++
	β-Lactoglobulin			10	++++
	Serum albumin			100	++++
	Milk albumin			100	++
	Histamine				++++

* +++++ indicates maximal contractions.

albumin (Table I). The observation that β -lactoglobulin reacted maximally in small doses indicated that the animal was sensitive to this protein rather than to a contaminant in the β -lactoglobulin. However, to exclude the possibility that the reactions induced by β -lactoglobulin might have been caused by albumin, the albumin content of the β -lactoglobulin was investigated by the quantitative method. Animals for these tests were sensitized with serum albumin, the serological equivalent of milk albumin. In one typical experiment slight but definite uterine contraction occurred

with 0.045 γ of protein N from serum albumin. On the other hand, maximal doses (1600 γ of protein N) of β -lactoglobulin did not induce any response. Moreover, maximal doses of β -lactoglobulin did not desensitize the tissue to minimal doses of either serum albumin or milk albumin. Therefore, the β -lactoglobulin contained less than 0.003 per cent of albumin.

The presence of β -lactoglobulin in the milk albumin was corroborated by the second experiment recorded in Table I. Four uterine strips from Guinea Pig 2, sensitized with β -lactoglobulin, were used. 10 γ of protein N from milk albumin did not contain enough β -lactoglobulin to induce a reaction (Strip 1). 100 γ of protein N from albumin elicited a submaximal response (Strip 2), but this dose did not contain enough of the contaminant to produce desensitization to subsequent reaction with 1 γ of protein N from β -lactoglobulin. On the other hand, the reaction induced by 1 γ of protein from β -lactoglobulin desensitized the tissue to 100 γ of protein N from milk albumin (Strip 3). In contrast to the results with milk albumin, a dose of 100 γ of protein N from serum albumin (Strip 4) did not elicit a response, further confirmation that the responses with large doses were not caused by the albumin *per se*.

The concentration of β -lactoglobulin in the milk albumin was determined in three experiments by the quantitative method. In one, the response induced by the β -lactoglobulin in a dose of the milk albumin containing 60 γ of protein N was about equivalent to that induced by 0.011 γ of protein N from the pure protein. This test indicated that the milk albumin contained about 0.018 per cent β -lactoglobulin. The other two tests showed 0.012 and 0.015 per cent.

An experiment designed to determine whether the milk albumin contained contaminants in addition to β -lactoglobulin is recorded in Table I. Guinea Pig 3 was sensitized with milk albumin. Two uterine strips from this animal were first desensitized with 10 γ of protein N from β -lactoglobulin. Subsequent tests were made at a dosage of 100 γ of protein N. Milk albumin elicited a response in Strip 1, and this reaction desensitized the tissue to serum albumin. In Strip 2, serum albumin induced a reaction subsequent to desensitization with β -lactoglobulin, but reaction with the two preceding preparations did not desensitize uterine tissue to the final test with milk albumin. These results showed that the milk albumin contained at least one contaminant in addition to β -lactoglobulin. The second contaminant was not identified, but later developments indicated that it was an immune globulin.

Experiments recorded in Table II were designed to determine whether the specificities of the isolated proteins were altered during isolation and to demonstrate their serological relationship to each other, to cow's milk, and to blood serum.

TABLE II
Relationship of Isolated Serum and Milk Proteins to Milk and Serum

Guinea pig No.	Sensitizing antigen	Uterine strip No.	Preparation added	Shock dose	Uterine contractions*
				<i>γ protein N</i>	
4	β -Lactoglobulin	1	Milk	100	++++
			β -Lactoglobulin	10	0
			Histamine		++++
5	Serum albumin	2	Bovine serum	100	0
			β -Lactoglobulin	1	++++
			Bovine serum	10	++++
		1	Milk albumin	10	0
			Histamine		++++
			Bovine serum	10	++++
		2	Serum albumin	10	0
			Histamine		++++
			Serum albumin	10	++++
		3	Bovine serum	10	0
			Histamine		++++
			Milk albumin	10	++++
6	Serum albumin	4	Bovine serum	10	0
			Histamine		++++
			Milk	100	++++
		1	Serum albumin	10	++++
			" "	10	++++
			" "	10	++++
		2	Milk	100	0
			Histamine		++++
			Milk	1000	++++
		3	Serum albumin	10	0
			Histamine		++++
			Serum albumin	10	++++
7	Bovine serum	4	Milk	1000	0
			Histamine		++++
			Serum albumin	10	++++
		1	Milk albumin	10	0
			Bovine serum	10	++++
			β -Lactoglobulin	10	0
		2	Milk albumin	10	++++
			Serum albumin	10	0
			Milk	10	++++
		3	Serum albumin	100	++++
			Milk albumin	100	++++
			Bovine serum	100	++++
8	Milk	1	β -Lactoglobulin	100	0
			Milk albumin	100	++++
			Serum albumin	100	0
			Milk	100	++++
			" albumin	10	++++
			Serum albumin	10	0
			Milk	10	++++
			Milk	10	++++

* +++++ indicates maximal contractions.

TABLE II—*Concluded*

Guinea pig No.	Sensitizing antigen	Uterine strip No.	Preparation added	Shock dose	Uterine contractions*
8	Milk	2		<i>γ protein N</i>	
			Serum albumin	10	++++
			Milk albumin	10	0
			Bovine serum	10	+

Uterine muscles from Guinea Pig 4, sensitized with β -lactoglobulin, reacted with milk but showed no sensitivity to bovine serum. This confirmed recorded observations (9–11) that β -lactoglobulin was distinct from any serum protein.

Guinea Pig 5 was sensitized with serum albumin. Individual strips from this animal contracted upon first addition of bovine serum, serum albumin, and milk albumin. Moreover, tissues sensitized to serum albumin were desensitized as effectively by milk albumin or whole serum as by serum albumin.

The experiment on Guinea Pig 6 showed that milk in adequate doses also produced desensitization to serum albumin. Because of the low concentration of albumin in milk, a dose of 1000 γ of protein N from milk was required for desensitization to subsequent tests with 10 γ of protein N from serum albumin.

Guinea pigs sensitized with alum-precipitated bovine serum regularly developed sensitivity to the albumin preparations, provided that the incubation period was sufficiently prolonged. Uterine strips from two animals sensitized with bovine serum and tested after an incubation period of 24 to 26 days showed no sensitivity to albumin, but three animals from the same group all showed sensitivity to albumin after a 90 day incubation period. Slow development of sensitivity to the albumin fraction of whole serum has been noted previously (12).

The experiment on Guinea Pig 7, Table II, shows the responses observed with β -lactoglobulin, milk albumin, and serum albumin in uterine muscle, sensitized with whole serum. β -Lactoglobulin failed to react at either the 10 or 100 γ of protein N dosage, further evidence of the distinction between this protein and components of blood serum. At 10 γ of protein N serum albumin reacted and produced desensitization to milk albumin. Residual antigens of bovine serum then induced a final maximal response. In Strip 2, milk albumin elicited a maximal response, subsequent to the negative test with β -lactoglobulin, and desensitized the tissue to serum albumin. However, the preceding tests did not desensitize the tissue to a final test with milk. The final response to milk showed that milk contained another protein, common also to bovine serum, in addition to and distinct from crystalline milk or serum albumin. Other workers (10, 11, 13, 14) have

shown that milk and serum contain serologically identical immune globulins.

Evidence of contamination of the milk albumin with protein, distinct from β -lactoglobulin, was derived from the results of tests conducted with 100 γ of protein N on uterine strips from Guinea Pig 7. Thus reaction with and desensitization to serum albumin did not produce desensitization to milk albumin. Hence, the second contaminant in the milk albumin was protein common to both milk and serum.

Guinea pigs sensitized with milk did not regularly develop sensitivity to the albumin fraction, even after a prolonged incubation period. This was probably due to a combination of the low concentration of albumin in milk and the weak sensitizing character of this protein. Lewis (15) and Doerr and Berger (16) showed that a large amount of strong antigen injected simultaneously with a small amount of weak antigen suppressed sensitization to the less active antigen. In the present experiments, four animals were sensitized with milk and only one exhibited significant sensitivity to albumin. However, the reactions of milk and serum albumins observed in uterine strips from this animal (Guinea Pig 8, Table II) confirmed the identity of these two proteins. In addition, the response elicited by bovine serum following desensitization with the two albumin preparations (Strip 2) confirmed the presence of the second protein common to milk and serum.

DISCUSSION

The crystalline milk albumin with the electrophoretic mobility of blood serum albumin, which has been isolated by Polis, Shmukler, and Custer (1), was shown to be serologically identical with serum albumin. This confirms a statement by Peskett (17) that normal milk contains a trace of protein serologically identical with serum albumin. At least one other protein of milk, common also to bovine serum, was detected but not identified. It was probably immune globulin identified by previous workers (10, 11, 13, 14).

Two distinct contaminants were detected in the milk albumin preparation. One was identified as β -lactoglobulin which was present in a concentration of about 0.015 per cent. The second contaminant was identifiable with some component of blood serum.

The β -lactoglobulin preparation did not contain a detectable trace of albumin and, in agreement with Palmer's report (9), was clearly distinct from any component of blood serum.

Because of the confusion of terms for the designation of the proteins of milk, it is difficult to correlate our findings with preceding work. From the results of gross anaphylaxis experiments, Wells and Osborne (13) concluded that lactoglobulin (immune globulin) was related to bovine serum

but that lactalbumin and serum albumin were two distinct proteins. Later, Palmer (9) showed that the fraction commonly labeled lactalbumin actually consisted largely of a readily crystallizable globulin, β -lactoglobulin (18). The residual protein of lactalbumin had the properties of a true albumin. However, even though the crystalline milk albumin may have been present in the lactalbumin fraction, it is unlikely that Wells and Osborne could have detected it by their method. They used the less sensitive gross anaphylaxis technique with intraperitoneal injection of the shock dose. Moreover, the incubation period in their experiments (18 to 20 days) was too short to permit optimal development of antibodies to the weak albumin antigen, particularly when accompanied by a large amount of the more potent β -lactoglobulin antigen.

SUMMARY

Crystalline bovine milk albumin was shown to be serologically identical with blood serum albumin. Bovine serum and normal milk contained at least one other protein common to both. In agreement with previous work, β -lactoglobulin was clearly distinct from components of bovine serum.

Two contaminants were detected in the crystalline milk albumin preparation. One was identified and quantitatively estimated as β -lactoglobulin. The second contaminant was identifiable with some component of bovine serum.

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ANTAGONISMS IN THE UTILIZATION OF D-AMINO ACIDS BY LACTIC ACID BACTERIA

III. INFLUENCE OF DL-METHIONINE SULFONE AND OXIDIZED CASEIN ON THE UTILIZATION OF D-METHIONINE*

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The growth-promoting activity of D-methionine for *Lactobacillus arabinosus* 17-5 was reported previously to be equal to that of L-methionine in a medium containing at least 1 γ per cent of pyridoxamine or 10 γ per cent of pyridoxal, but negligible in the absence of these vitamins (2). The inactivity of D-methionine for *L. arabinosus* which had been found earlier (3, 4) was attributed to the pyridoxamine and pyridoxal deficiencies of the experimental media employed in these studies. D-Methionine activity for *L. arabinosus* had also been shown to be negligible in a pyridoxamine-rich medium containing a hydrolysate of oxidized casein (5), and it was suggested, therefore (2), that oxidized methionine from such a hydrolysate might interfere with the utilization of D-methionine by this bacterium.

The present report is concerned with the effect of oxidized casein hydrolysate and the oxidized forms of methionine, DL-methionine sulfone, and DL-methionine sulfoxide on the utilization of D-methionine by *L. arabinosus*. A comparable study of the effect of DL-methionine on D-methionine utilization has been reported previously (6).

EXPERIMENTAL

The culture of *L. arabinosus*, the basal medium, and the experimental methods were the same as those described previously (2). DL-Methionine sulfoxide¹ and DL-methionine sulfone² were prepared by the methods of Toennies and Kolb (7, 8). Oxidized casein was prepared by the method of Toennies (9) and hydrolyzed by refluxing 18 hours in 30 per cent sulfuric acid. The sulfuric acid was removed from the hydrolysate by neutralization with barium hydroxide. The experimental medium was

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¹ Purified by recrystallization from 65 per cent ethanol.

² Purified by recrystallization from water.

modified in the different experiments by additions of DL-methionine sulfide, DL-methionine sulfone, and oxidized casein hydrolysate, and the response of *L. arabinosus* to L-methionine,² DL-methionine,³ and D-methionine⁴ was determined titrimetrically. The results are given in Tables I and II and Figs. 1 and 2.

DISCUSSION

DL-Methionine sulfone and oxidized casein hydrolysate were found to inhibit the growth of *L. arabinosus* in the basal medium supplemented

TABLE I
Calculated Activities of DL-Methionine, D-Methionine, and DL-Methionine Sulfoxide for *L. arabinosus* 17-5*

Addition to basal medium†	Test sample		
	DL-Methionine	D-Methionine	DL-Methionine sulfoxide
	per cent	per cent	per cent
None.....	102 (1.8)	101 (3.1)	103 (1.3)
DL-Methionine sulfone, 60 mg. %.....	59 (6.6)	18 (23)	25 (6.8)
“ “ 240 “ %.....	57 (2.9)	18 (37)	25 (11)
Oxidized casein hydrolysate, 0.5 %.....	62 (6.1)	11 (7.5)	58 (13)
“ “ “ 1.0 %.....	52 (1.5)	5 (13)	18 (20)

* Per cent activity =

$$\frac{100 \times \text{molar concentration found as L-methionine}}{\text{molar concentration of substance tested}}$$

Each value is the average of those found for the fourteen dosage levels tested. The values in parentheses are the mean per cent deviations from the means. The relatively high mean deviations found for D-methionine were the result of pronounced downward “drifts,” while those for DL-methionine sulfoxide were the result of upward “drifts.”

† Final concentrations given.

with L-methionine at final concentrations less than 5 γ per ml. and to reduce the relative activities of DL-methionine and D-methionine (Table I). DL-Ethionine was shown previously to reduce the relative activities of DL-methionine and D-methionine for *L. arabinosus* in a similar manner (6). It seems likely, therefore, that the latter compounds may ordinarily be converted enzymatically to L-methionine by *L. arabinosus* and that the efficiency of these processes may be reduced in the presence of competitive inhibitors. That oxidized casein hydrolysate is somewhat more effective than DL-methionine sulfone alone in depressing the utilization of D-methionine by *L. arabinosus* is evident from the data given in Table I. It may be seen that a supplement of 1 per cent of oxidized casein

* Product described previously (2).

hydrolysate, which supplies a calculated final concentration of 34 mg. per cent of L-methionine sulfone, is considerably more effective in this respect

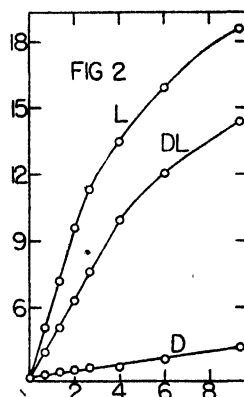
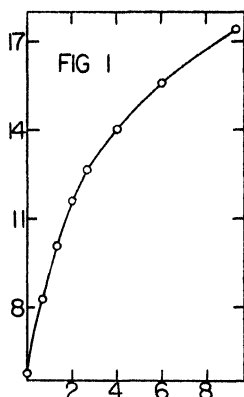


FIG. 1. Response of *L. arabinosus* to L-methionine, DL-methionine, and D-methionine in the basal medium. The experimentally determined values plotted for each of the three methionine forms fall within the diameters of the circles shown. The values on the horizontal scale represent the concentration of methionine in micrograms per ml. The values on the vertical scale represent ml. of 0.01 N NaOH required to titrate 1 ml. of culture.

FIG. 2. Same as Fig. 1, except that the basal medium was supplemented with 1 per cent oxidized casein hydrolysate.

TABLE II
*Acid Produced by L. arabinosus in Response to Additions of DL-Methionine and DL-Methionine Sulfoxide**

Sample added per tube $\mu \times 10^{-3}$	Sample	
	DL-Methionine	DL-Methionine sulfoxide
	ml.	ml
0	5.91	5.99
1	8.33	8.13
3	11.63	11.59
6	14.02	14.25
14	18.73	18.67

* Calculated as ml. of 0.01 N NaOH to titrate 1 ml. of final culture.

than final concentrations of DL-methionine sulfone of up to 240 mg. per cent.

The remarkable similarity of the response of *L. arabinosus* to L-methionine, DL-methionine, and D-methionine in the basal medium is illustrated in Fig. 1, and the effect of adding oxidized casein hydrolysate to the medium

is shown in Fig. 2. It may be noted that the response to D-methionine was almost negligible in the medium with oxidized casein hydrolysate (Fig. 2) but that the sensitivity to L-methionine was not decreased under these conditions. It appears, therefore, that oxidized casein hydrolysate may be used without ill effect in media employed for the determination of methionine with *L. arabinosus*, provided that D-methionine is absent or only L-methionine is to be estimated.

It is evident from the data given in Table II that DL-methionine sulfoxide did not inhibit the growth of *L. arabinosus*, but instead had nearly the same activity as DL-methionine in promoting the growth of this bacterium.⁴ The possibility that the sulfoxide was reduced to methionine in autoclaving with the basal medium was excluded by diluting a 50 per cent ethyl alcohol solution (assumed to be sterile) of the sulfoxide with sterile water and adding the mixture aseptically to the previously sterilized assay tubes containing the sterile test medium. The results under these conditions were the same as with concurrent sterilization of medium and test sample. The data given in Table I show that the relative activity of DL-methionine sulfoxide was considerably reduced in the media containing DL-methionine sulfone and oxidized casein hydrolysate. It seems likely, therefore, that the sulfoxide is converted to methionine in its utilization by *L. arabinosus* and that the inhibitory substances partially block this conversion.

SUMMARY

D-Methionine and DL-methionine sulfoxide were found to have growth-promoting activities for *Lactobacillus arabinosus* equal to that of L-methionine under the given test conditions. DL-Methionine sulfone or oxidized casein hydrolysate added to the basal medium suppressed the utilization of D-methionine and DL-methionine sulfoxide.

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⁴ It is of interest that DL-methionine sulfoxide may be substituted for methionine in the nutrition of the rat (10).

FORMATION OF FORMATE AND LABILE METHYL GROUPS FROM ACETONE IN THE INTACT RAT*

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Price and Rittenberg (1) have reported the ability of the rat to utilize acetone, and Borek and Rittenberg (2) have implicated the liver in this process. These investigators have demonstrated the conversion of acetone to cholesterol and indicate that this may occur, at least in part, via acetate.

The possibility has been presented that acetone is converted to "acetate"¹ by an oxidative process in which "formate"² is formed from one of the methyl groups. Since it had been shown (3-5) that formate contributes to the β -carbon of serine, it was possible to test this hypothesis by determining whether the carbon of acetone methyl groups was similarly utilized in serine formation. This was found to be the case. Under the same conditions, relatively little of the methyl and methylene carbon of acetoacetate and acetate administered to rats was utilized in this manner. It is apparent from these results that formate is not formed from acetone via carboxylation to acetoacetate or as a metabolite of acetate.

It has also been demonstrated that the methyl carbon of acetone contributes to the methyl groups of choline and methionine. This has suggested the possibility that a 1-carbon compound arising from acetone is used to synthesize labile methyl groups. The problem of methyl synthesis has been investigated by Sakami and Welch (6) using labeled formate, and it has been demonstrated that choline and methionine methyl groups are synthesized from this compound.

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An abstract of this paper was submitted on January 12, 1950, to the Society of Biological Chemists for presentation at the 34th annual convention of the Federation of American Societies for Experimental Biology. The findings also were discussed by Dr. Harland G.³ Wood in a Harvey Lecture, New York, February 16, 1950.

The radiocarbon used in these experiments was obtained on allocation from the United States Atomic Energy Commission.

¹ "Acetate" is used to indicate either acetate itself or a 2-carbon intermediate of its metabolism.

² "Formate" is used to indicate either formate itself or a 1-carbon intermediate of its metabolism.

EXPERIMENTAL

The acetone employed in these experiments was labeled with an activity¹ of 7.40×10^4 c.p.m. per mg. of methyl carbon. It was prepared by the pyrolysis of C¹⁴-methyl-labeled barium acetate (7) and purified by distillation over acid permanganate solution and conversion into the *p*-nitrophenylsemicarbazone (8). Acetone was recovered from its derivative by distillation with 1 M sulfuric acid. The acetone was 100 ± 2 per cent pure, as determined by the salicylaldehyde method (9).

In Experiment 1 six male albino rats were fasted for 48 hours, after which they weighed from 120 to 150 gm. Each was given doses of 2 ml. of 2.5 M glycine solution by stomach tube and 0.25 ml. of 0.30 M C¹⁴-methyl-labeled acetone solution subcutaneously per 100 gm. The latter dose was repeated at the end of the 3rd, 6th, and 10th hours. Two of the rats were kept in a metabolism cage which permitted quantitative collection of the respiratory CO₂.

The C¹⁴ activities of consecutive hourly samples of the respiratory CO₂ are shown in Table I. The acetone was well metabolized; during the 14 hours of the experiment 53 per cent of the acetone methyl carbon administered to the rats in the metabolism cage was recovered in the CO₂. This is in agreement with the observations of Price and Rittenberg (1).

After 14 hours, the abdominal viscera were removed under sodium amytal anesthesia and pooled. The livers were immediately homogenized with 100 ml. of acetone, filtered, and stirred for 12 hours with 100 ml. of a mixture of 2 volumes of alcohol and 1 of ether. This extraction was repeated twice. The acetone and alcohol-ether solutions were combined and evaporated to dryness *in vacuo*. The residue, which contained the liver phosphatides, was extracted with a small amount of acetone and saved for the isolation of choline.

Glycogen was then extracted from the liver powder by homogenizing it with 50 ml. of 5 per cent trichloroacetic acid solution and stirring the resulting suspension for a half hour. This procedure was repeated twice. The extract was saved for the isolation of glycogen. The residue was washed with acetone, dried in air, and set aside for the isolation of serine.

After removing the contents of the gastrointestinal tracts, the phospholipides were extracted from the remainder of the abdominal viscera by the procedure described above and combined with the liver phosphatides. Choline was isolated from this material as the reineckate (10) and purified

¹ The activity of all samples except the tetramethylammonium iodide was determined by conversion into barium carbonate and counting with an end window Geiger counter. The tetramethylammonium iodide was plated on stainless steel planchets and counted directly, but for purposes of comparison the results have been calculated and expressed as the activity determined on barium carbonate.

by conversion into the chloroplatinate (11). This was recrystallized by precipitation from aqueous solution by means of alcohol and analyzed (platinum found 3.16 per cent, calculated 3.17 per cent). Platinum was then removed (12) and the choline was degraded by oxidation to trimethylamine (13, 11). The amine was trapped in a 5 per cent solution of methyl iodide in alcohol, cooled in dry ice-cellosolve. The solution was left overnight at room temperature and evaporated to dryness *in vacuo*. The tetramethylammonium iodide that remained was dissolved in a small amount of hot water and precipitated with alcohol (iodine found 63.4 per cent, calculated 63.1 per cent). It possessed significant activity (Table

TABLE I

Excretion of C¹⁴ in Respiratory CO₂ after Administration of C¹⁴-Methyl-Labeled Acetone and Methyl- and Methylene-Labeled Acetoacetate**

Hour	Activity of respiratory carbon dioxide				Hour	Activity of respiratory carbon dioxide			
	Specific activity†		Total activity‡			Specific activity†		Total activity‡	
	Acetone	Acetoacetate	Acetone	Acetoacetate		Acetone	Acetoacetate	Acetone	Acetoacetate
1st	121	620	17,400	130,000	8th	561	624	65,600	56,200
2nd	187	474	28,800	71,200	9th	614	387	70,000	35,100
3rd	259	233	33,800	26,500	10th	619	179	66,000	23,400
4th	290	828	34,400	121,000	11th	588	896	66,200	135,000
5th	398	526	46,600	84,600	12th	674	748	70,800	118,000
6th	454	273	53,200	41,700	13th	700	344	71,400	48,300
7th	487	986	57,600	164,000	14th	644	221	62,800	26,800
Total.....								745,000	1,080,000

* The total activities administered as acetone and acetoacetate were 1.41×10^6 c.p.m. and 1.95×10^6 c.p.m. respectively.

† Counts per minute per mg. of carbon.

‡ Counts per minute.

II, Experiment 1) which was not changed on recrystallization of the compound from a solution of ethanol and water.

Serine was isolated from the liver protein by a modification of the procedure of Stein *et al.* (14) for the preparation of this amino acid from silk. The protein was boiled for 24 hours with 160 ml. of 20 per cent HCl. Basic amino acids were removed by precipitation with phosphotungstic acid according to the procedure of Van Slyke (15). Excess phosphotungstic acid was then extracted from the filtrate with amyl alcohol and dicarboxylic amino acids were isolated by the method of Cannan (16). Approximately 12 gm. (air-dried weight) of Amberlite IR-4B were re-

quired. The hydrolysate remaining after the removal of the basic and dicarboxylic amino acids was concentrated *in vacuo* to a small volume. Tyrosine precipitated and was removed by filtration. The filtrate was concentrated *in vacuo* to 10 ml., and 3 gm. of 5-nitronaphthalene-1-sulfonic acid were dissolved into it. The solution was seeded with glycine 5-nitronaphthalene-1-sulfonate and allowed to stand at 6° for 2 days. The precipitate was removed by filtration and washed with 2 ml. of a cold 5 per cent solution of the sulfonic acid. 1 ml. of monomethyl cellosolve and 3 gm. of azobenzene-*p*-sulfonic acid were added to the combined filtrate and wash water. The mixture was heated to dissolve the sulfonic acid, cooled to 6°, seeded with L-alanine azobenzene-*p*-sulfonate, and kept

TABLE II

C¹⁴ Activity of Compounds Isolated from Rats Receiving C¹⁴-Methyl-Labeled Acetone, Acetic Acid, or Acetoacetic Acid

Experiment No.	C ¹⁴ Compound administered	Compound degraded	Labeling of carbon fractions*			
			CH ₃	COOH or 3,4 position†	α or 2,5 position†	β or 1,6 position†
1	Acetone	Serine		8	15	420
2	Acetate	"		5	2	6
4	Acetoacetate	"		7	4	15
1	Acetone	Glycogen		399	340	447
2	Acetate	"		157	177	176
1	Acetone	Choline	150			
3	"	"	224			
4	Acetoacetate	"	7			
3	Acetone	Methionine	197			

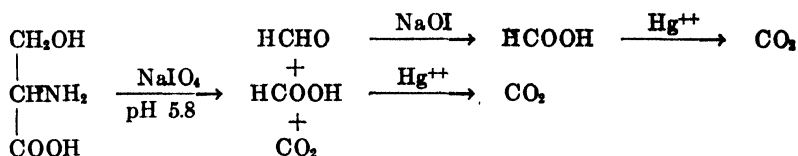
* Activity in counts per minute per mg. of carbon.

† Position in the glucose unit of the glycogen.

at this temperature for 2 days. The L-alanine sulfonate was removed by filtration and washed with 2 ml. of cold, saturated, azobenzene-*p*-sulfonic acid solution. The filtrate and washings were combined, diluted to 250 ml., and freed from the sulfonic acids with Amberlite IR-4B by the usual procedure. Approximately 40 gm. (air-dried weight) of resin were required. The deacidified solution was concentrated *in vacuo* to 10 ml., 0.5 ml. of monomethyl cellosolve was added, and 3 gm. of *p*-hydroxyazobenzene-*p*'-sulfonic acid were dissolved in this solution with heating. The mixture was then cooled, seeded with L-serine *p*-hydroxyazobenzene-*p*'-sulfonate, and kept at 6° overnight. The precipitate obtained in this manner was dissolved in 25 ml. of hot water. The material that repre-

precipitated on standing at room temperature for 24 hours was removed and discarded. The filtrate was concentrated to 5 ml. and allowed to stand at 6° overnight. L-Serine *p*-hydroxyazobenzene-*p'*-sulfonate precipitated in glistening plates. It was purified by recrystallizing it twice from small amounts of water and dried *in vacuo* over CaCl₂. It decomposed at 206° simultaneously with a sample of pure L-serine *p*-hydroxyazobenzene-*p'*-sulfonate; a mixture of the two substances decomposed at the same temperature. No ninhydrin-reacting substance other than serine could be detected by a 26 cm. phenol-water paper chromatogram.

The serine derivative possessed considerable radioactivity. To determine the position of this activity in the serine, it was degraded by the scheme shown in the accompanying diagram. This procedure was based on a study of the oxidation of serine with periodate by Sprinson and Chargaff (17).



0.201 gm. (0.5 mm) of serine *p*-hydroxyazobenzene-*p'*-sulfonate monohydrate⁴ was dissolved in 2.34 ml. of 0.214 N NaOH. 4 ml. of 0.5 M sodium phosphate buffer, pH 5.8, were added and the solution was aerated to remove preformed CO₂. 3 ml. of CO₂-free 0.5 M NaIO₄ were then introduced, and the mixture was aerated for 1 hour to remove the CO₂ formed from the serine carboxyl carbon. The yield of CO₂, which was trapped in sodium hydroxide solution and determined gravimetrically, was 94 per cent.

In order to remove iodate and periodate from the solution, 5 ml. of 1 M SrCl_2 were introduced and the pH was raised to 6 by the addition of 2 ml. of 1.81 N NaOH. The solution was then mixed and kept at 6° for 5 hours. The precipitate, which consisted of strontium iodate, periodate, phosphate, and *p*-hydroxyazobenzene-*p'*-sulfonate, was removed by centrifugation and washed twice on the centrifuge with small volumes of cold water. The centrifugate and wash water were combined, adjusted to pH 3 with glacial acetic acid, and boiled and aerated to remove preformed CO_2 . 25 ml. of a CO_2 -free solution of mercuric chloride in acetate buffer

* L-Serine *p*-hydroxyazobenzene-*p'*-sulfonate, dried over CaCl_2 , is a monohydrate. A sample of this material lost 5.00 per cent in weight (theory for 1 H_2O , 4.49 per cent) when heated *in vacuo* over P_2O_5 at 100° . It then contained 3.63 per cent "periodate- $\text{NH}_2\text{-N}$ " (18) (theory for $\text{C}_{15}\text{H}_{17}\text{O}_7\text{N}_2\text{S}$, 3.65 per cent).

(19) were then added, and boiling was continued for 1 hour to oxidize the formate to carbon dioxide. This procedure does not oxidize formaldehyde. The CO_2 was removed by aeration and trapped in sodium hydroxide solution. The yield of CO_2 derived from the serine α -carbon was 84 per cent.

The solution was filtered from mercurous chloride and distilled to a volume of 10 ml. The distillate, which contained the formaldehyde, was cooled to 6° . 20 ml. of 1 *N* NaOH and 20 ml. of 0.1 *N* I_2 , both also at 6° , were added and the solution was allowed to stand at this temperature for 10 minutes. Formaldehyde was oxidized to formate in this procedure. The solution was then acidified to pH 4 with 2 *M* sulfuric acid and excess iodine was reduced with 0.1 *N* thiosulfate. The formate was oxidized to CO_2 as described above. The yield of CO_2 formed from the serine β -carbon was 72 per cent.

The CO_2 samples were converted into BaCO_3 for determinations of radioactivity. The results of the isotope analyses are shown in Table II (Experiment 1).

Almost all of the activity was located in the β -carbon atom.

Liver glycogen was isolated from the trichloroacetic acid extracts of liver. It was purified by alcohol precipitation from a dilute lithium chloride solution (20) and degraded (21). The results of the isotope analyses are recorded in Table II (Experiment 1). The isotope activity of the 1,6-carbons was significantly higher than that of the 2,5-carbons.

Experiment 2 was similar to Experiment 1 except that the doses of 0.25 ml. of acetone solution were replaced by 0.50 ml. of 0.15 *M* C^{14} -methyl-labeled acetate containing 7.40×10^4 c.p.m. per mg. of methyl carbon. The fasted weights of the four rats used were 110 to 150 gm. Liver serine and glycogen were isolated and degraded as before. The results of the isotope analyses of the glycogen and serine carbon fractions are shown in Table II (Experiment 2). The serine contained no appreciable activity in any position. The 1,6 and 2,5 positions of the glycogen were equally labeled.

Experiment 3 was designed as an additional test of the conversion of the acetone methyl carbon to labile methyl groups. It differed from Experiments 1 and 2 in that the rats were not given glycine, since there was no desire to isolate glycogen, and only the choline and methionine methyl groups were studied. The three rats used had weights of 185 to 190 gm. after fasting. At the end of the experiment, the contents of the gastrointestinal tracts were removed and the abdominal viscera were pooled for the isolation of choline and methionine methyl groups.

Choline was isolated as in Experiment 1, but was purified by conversion

into the mercuric chloride double salt (22). The mercury was removed with H_2S , and the choline was converted into tetramethylammonium iodide, as described in Experiment 1 (iodine 62.2 per cent). The tetramethylammonium iodide possessed moderate activity which was not affected by recrystallization from ethanol-water (Table II, Experiment 3).

The visceral protein was prepared for the demethylation of its methionine by extraction with acetone, alcohol-ether, trichloroacetic acid, and again with acetone, and dried. The protein was then hydrolyzed by heating it with 6 N HCl for 20 hours, and the hydrolysate was concentrated *in vacuo* and demethylated with hydriodic acid (23, 24). The methyl iodide formed was converted into tetramethylammonium iodide (24) (iodine 63.8 per cent). This possessed moderate radioactivity (Table II, Experiment 3) which remained unchanged on recrystallization of the compound from a mixture of alcohol and water. The results of Experiment 3 thus indicate clearly that the methyl carbon of acetone is converted to labile methyl groups, confirming the results of Experiment 1 in this respect.

Experiment 4 was similar to Experiment 1, except that the doses of acetone solution were replaced with 0.27 ml. of 0.27 M C^{14} -methyl- and methylene-labeled sodium acetoacetate containing 1.09×10^5 c.p.m. per mg. of labeled carbon, prepared by the procedure of Sakami, Evans, and Gurin (25). The weights of the rats upon fasting were 130 to 135 gm.

The results of the isotope analyses of the serine and choline are shown in Table II. They contained relatively slight activity.

The hourly excretion of C^{14} in the respiratory CO_2 is shown in Table I. Approximately 55 per cent of the administered activity was recovered in the CO_2 .

DISCUSSION

The experiments reported in this communication demonstrate that in the intact rat the β -carbon of serine and the methyl groups of choline and methionine may be formed from acetone methyl carbon. These groups are also formed from formate (3-6). The fact that formate and acetone contribute to the formation of the same carbon moieties indicates that acetone is metabolized via formate or some substance derived from it. Comparison of the extent to which acetone and formate carbon are incorporated into the serine indicates that this is an important pathway of acetone metabolism. The activity of the β -carbon of serine formed from acetone is comparable to that formed from an approximately equivalent amount of formate (3) possessing roughly twice the activity of one of the methyl carbons. The conditions of these two experiments were

identical, except that the formate was injected intraperitoneally in two equal doses, whereas the acetone was administered subcutaneously in four.

The mechanism of this formation of "formate" does not involve a preliminary carboxylation of acetone to acetoacetate. If this were the case, C¹⁴ administered as methyl- and methylene-labeled acetoacetate would label the β -carbon of serine similarly to that administered as acetone. Very little activity was actually found in the serine.

The experiments of Price and Rittenberg (1) and Borek and Rittenberg (2) indicate that acetate is also an important product of acetone metabolism. The latter investigators have found that the rate of introduction of the deuterium of labeled acetone into cholesterol in liver slices is comparable to that of labeled acetate and is diminished by the presence of non-isotopic acetate during the incubation. Price and Rittenberg (1) have concluded from the extent of incorporation of the C¹⁴ of methyl-labeled acetone into the acetyl groups of acetylphenylaminobutyric acid in the intact rat that about half of the acetone was converted to acetate.

These metabolites, "acetate" and "formate," are produced from acetone separately. As no significant activity was introduced into the liver serine from the C¹⁴ of methyl-labeled acetate, "formate" cannot be formed via "acetone." The production of "acetate" as a major metabolite of "formate" is equally improbable. It is therefore reasonable to hypothesize that acetate is catabolized via a 2- and 1-carbon cleavage in which "formate" and "acetate" are formed, although it is also possible that they are formed by different mechanisms. This type of cleavage is consonant with the manner in which the C¹⁴ of methyl-labeled acetone labels the liver glycogen. There is significantly more isotope in the 1,6 than in the 2,5 positions (Table II). That this is the expected isotope distribution is indicated by the following considerations. First, C¹⁴ administered as formate (3) labels the liver glycogen in a manner similar to that obtained with methyl-labeled acetone (Table II) in that the 1,6-carbons are significantly more active than the 2,5-carbons. Second, methyl-labeled acetate yields equal labeling in the 1,6 and 2,5 positions (Table II) (26). Thus, if in addition to formate acetate also introduces labeling into the glycogen, the higher activity of the 1,6 in comparison with the 2,5 positions would not be altered.

The significance of acetone as an intermediate in acetoacetate metabolism is under investigation. The rate of acetone oxidation as indicated by the appearance of its C¹⁴ in the respiratory CO₂ (Table I) (1) is sufficiently rapid to indicate the possible importance of this pathway. The spontaneous decarboxylation of acetoacetate is, however, rather slow and,

unless catalyzed by a decarboxylase analogous to that of *Clostridium acetobutylicum* (27, 28), would account for only a small disappearance of acetoacetate.

The low incorporation of the C¹⁴ of acetoacetate into the serine carbon is not an indication that the amount of this substance metabolized via acetone is insignificant under the conditions of our experiment. The dilution of the labeled compound by endogenous acetoacetate may have been considerable in these fasted animals.

The author wishes to acknowledge the valuable suggestions of Dr. H. G. Wood.

SUMMARY

Methyl-labeled acetone has been administered to rats and its conversion to glycogen, the β -carbon of serine, and the labile methyl groups of choline and methionine has been demonstrated. The mechanisms of these transformations do not involve the carboxylation of acetone to acetoacetate, but are believed to occur by oxidation of the acetone to "acetate" and "formate" with subsequent synthesis of serine and labile methyls. The possible importance of acetone as an intermediate of fat metabolism is considered.

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SYNTHESIS OF LABILE METHYL GROUPS BY THE RAT IN VIVO AND IN VITRO*

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The intact rat has been shown to be capable of deriving the methyl groups of methionine and choline from the methyl carbons of acetone (1). As these carbons also contribute to the "formate" pool from which the β -carbon of serine is formed (1), the possibility has been presented that a 1-carbon compound is an intermediate in the formation of labile methyl groups from acetone. In preliminary experiments, this hypothesis was tested by determining the C^{14} activity of methionine methyl carbon isolated from rats to which labeled formate had been given in another experiment. The presence of significant radioactivity in this carbon demonstrated the ability of the intact rat to generate the labile methyl group from formic acid. Accordingly, at least a part of the labile methyl supply obtained from the methyl carbons of acetone must be derived via this formate¹ route.

The demonstration of the synthesis *in vivo* of labile methyl groups from formate is consonant with certain observations of du Vigneaud and his coworkers (2). They noted a small but significant entrance of deuterium into the methyl groups of choline, when rats were fed for 3 weeks "a diet adequate in labile methyl groups" (20 per cent casein) and the deuterium concentration of the body water was maintained at about 3 atom per cent. These results were not attributed to a slow labilization

* An abstract of this paper was submitted on January 12, 1950, to the Society of Biological Chemists for presentation at the 34th annual convention of the Federation of American Societies for Experimental Biology in April (Welch, A. D., and Sakami, W., *Federation Proc.*, 9, 245 (1950)).

† Aided by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council and by assistance of the Elisabeth Severance Prentiss Foundation, and also by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

The radiocarbon used in these experiments was obtained on allocation from the United States Atomic Energy Commission.

¹ "Formate" is used to indicate either formate itself or a 1-carbon intermediate of its metabolism.

of methyl-bound hydrogen, but were considered probably to reflect the synthetic activity of the bacteria of the intestine. In fact, the dietary indispensability of the labile methyl group is generally considered to have been firmly established.

The ability of the tissues of the rat to synthesize labile methyl groups now has been demonstrated by the use of liver slices *in vitro*. Incubation with C^{14} -formate led to the incorporation of significant activity into the methyl groups of choline and methionine.

EXPERIMENTAL

In the first experiment four rats were used which had been fed, for 5 days prior to and during the experimental period, a synthetic amino acid diet rich in methionine (1.25 per cent) and containing no glycine, serine, cystine, choline, or ethanolamine.² The animals weighed from 101 to 119 gm. at the start of the experiment. They were given, per 100 gm. of body weight, subcutaneous injections of 0.20 ml. of 0.15 M sodium formate solution at the beginning of the 1st hour and 0.15 ml. per 100 gm. at the beginning of each succeeding hour for a total of 10 hours. The formate contained 9.6×10^6 c.p.m. per mm.³ The animals were killed at the end of the 10th hour. The contents of the gastrointestinal tracts were discarded and the combined abdominal viscera (with the exception of the livers, which had been used in another experiment) were homogenized with acetone, extracted successively with ether-alcohol, trichloroacetic acid, and acetone, and dried. The dry protein was demethylated with boiling hydriodic acid (sp. gr. 1.7) (4, 5) and the methyl iodide formed was converted into tetramethylammonium iodide with trimethylamine and analyzed (iodine found 62.6 per cent, theory 63.1 per cent). The activity of the tetramethylammonium iodide, which was not altered by recrystallization, corresponded to 354 c.p.m. per mg. of methionine methyl carbon. Similar results were obtained in two rats given radioactive sodium formate subcutaneously every 2 hours for fifteen injections.

² This experiment was designed primarily to test the formation of the α -carbon of glycine from formate and only incidentally employed for the study of the synthesis of labile methyl groups. The diet employed was that of du Vigneaud *et al.* (3) with the following modifications: 1.25 per cent DL-methionine was added and the dextrin level was raised to 28.4 per cent. Glycine, serine, and cod liver oil were omitted. B vitamins were mixed with the diet in the following quantities per 100 gm. of diet: thiamine 1 mg., riboflavin 2 mg., niacin 10 mg., pyridoxine 1 mg., Ca pantothenate 5 mg., inositol 40 mg.

³ The activity of the formate was determined by conversion into barium carbonate and counting with an end window Geiger counter. The tetramethylammonium iodide samples were plated on stainless steel planchets and counted directly, but for purposes of comparison the results have been calculated and expressed as the activities determined on barium carbonate.

In an experiment carried out *in vitro*, approximately 6 gm. of rat liver slices were incubated for 4 hours in 25 ml. of the medium described by Winnick *et al.* (6) with an added 0.003 per cent MnSO_4 , in an atmosphere of 95 per cent O_2 -5 per cent CO_2 . Incubation was briefly interrupted and the following total additions were made in divided doses at 0, 1, 2, and 3 hours: sodium formate (0.63 ml., 0.3 M, 8.8×10^6 c.p.m.); homocysteine (40 mg., crystalline, as a solid); dimethylaminoethanol (0.8 ml., 0.14 M); specially purified folic acid⁴ (40 γ , 0.2 ml.); crystalline vitamin B_{12} (4 γ , 0.2 ml.). At the end of the 4th hour, the experiment was terminated. Additions of 250 mg. of DL-methionine, 25 ml. of concentrated hydrochloric acid, and 5 ml. of non-isotopic formic acid were made, and the formic acid was removed by steam distillation. Analysis of this formic acid indicated that, of the radioactive formic acid added to the slices, 22 per cent was recoverable as unmodified formate. Hydrochloric acid was then added to the liver residue to give a concentration of approximately 6 M and the mixture was refluxed for 20 hours. Methionine, isolated by the procedure of Hill and Robson (7), continued to lose activity through five recrystallizations from aqueous ethanol. Accordingly, the five times recrystallized material was heated with hydriodic acid (4, 5) and the methyl iodide obtained was recovered as tetramethylammonium iodide (iodine 62.8 per cent). The activity of the tetramethylammonium iodide corresponded to an activity of 540 c.p.m. per mg. of methionine methyl carbon; the activity was not affected by recrystallization.

To determine whether the isotopic activity that contaminated the methionine yielded methyl iodide, the crude residues from the methionine crystallizations were demethylated with hydriodic acid (4, 5) and the methyl iodide was converted into tetramethylammonium iodide (iodine 63.1 per cent). The isotopic activity of the tetramethylammonium iodide corresponded to an activity of 540 c.p.m. per mg. of methionine methyl carbon; the activity was not affected by recrystallization. This agreement between the radioactivity of the methyl iodide derived from the five times recrystallized methionine and that of the methyl iodide obtained from the residues of the purifications is evidence that the radioactive substance that contaminated the methionine did not interfere in the determination of the activity of the methionine methyl.

Choline chloride (approximately 50 mg.) was added to the residues from the methionine isolation. After evaporation to dryness, the choline was extracted with absolute ethanol and was precipitated as the reineckate. Following solution in 50 per cent acetone, the reineckate was cleaved by the procedure of Kapfhammer and Bischoff (8). The choline chloride

⁴ The folic acid was supplied by the Lederle Laboratories Division, American Cyanamid Company, through the courtesy of Dr. T. H. Jukes; it was prepared from recrystallized magnesium folate.

remaining in the filtrate was converted into the chloroplatinate, and from this choline chloride was regenerated by the method of Dudley (9). The choline was then oxidized to trimethylamine (10) and with methyl iodide was converted into tetramethylammonium iodide (iodine 62.8 per cent) (1). The activity of the tetramethylammonium iodide corresponded to an activity of 530 c.p.m. per mg. of choline methyl carbon; the activity was not affected by recrystallization. The demonstration of radioactivity in choline offers independent evidence for the validity of the conclusion to be drawn from the study of methionine; namely, that labile methyl groups were synthesized from formate.

DISCUSSION

These results demonstrate the ability of the rat to synthesize labile methyl groups. Normally, in rats ingesting relatively large amounts of compounds containing these groups, synthesis appears to contribute negligibly to the methyl groups of tissue choline and creatine. Thus, when rats were fed deuteriomethylmethionine over a period of 14 weeks, the concentration of deuterium in the methyl groups of the choline and creatine from the tissues rose to 85 per cent of the rats fed methionine (11). When L-methionine labeled with C^{14} and deuterium was fed for 4 days, the ratio of deuterium to C^{14} in the methyl groups of the choline and creatine was found, within experimental error, to be the same as that in the methyl groups of the dietary methionine (12). Rats maintained on heavy water for 3 weeks showed only a small deuterium content in their choline methyl groups (2). It should be noted that in all these experiments preformed labile methyl groups were supplied to the animals by diets containing at least 0.68 per cent L-methionine.

There are indications, however, that under certain conditions the biosynthesis of methyl groups may be accelerated. Thus several investigators have noted that rats given homocystine in lieu of methionine occasionally grow without any apparent source of methyl groups (3, 13-17). Bennett and her collaborators (13-16) have reported that a substance present in "liver extract, Lilly," permitted growth to occur more consistently under such circumstances. More recently, Bennett (17) has presented evidence indicating that for growth under these conditions folic acid is also required. Schaefer, Salmon, and Strength have noted (18) that diets containing choline in amounts insufficient to prevent fatty infiltration of the liver or renal hemorrhages in rats were made protective by the addition of concentrates of vitamin B_{12} or of the crystalline vitamin. Particularly striking was the effect on weanling rats of vitamin B_{12} added to a choline-free amino acid diet containing homocystine in

lieu of methionine; under these conditions slow growth was obtained, while, in the absence of the vitamin, death from renal hemorrhages occurred within a few weeks (Stekol and Weiss (19)).

In preliminary experiments, liver slices obtained from rats deficient in either folic acid or vitamin B₁₂ were incubated with homocysteine and C¹⁴-formate, but methionine was not isolated. Rather, the incubation mixture was freed of residual formic acid by steam distillation, dried, and subjected to direct hydrolysis with hydriodic acid.

Methyl iodide formed in this manner was converted to tetramethylammonium iodide, the activity of which was not altered by recrystallization. Addition *in vitro* of crystalline vitamin B₁₂ did not increase the activity of tetramethylammonium iodide prepared from the incubated liver slices of rats which had ceased to grow on a diet designed to produce a deficiency of the vitamin. On the other hand, addition of folic acid doubled the specific activity of the methyl iodide obtained from liver slices of folic acid-deficient rats. Since the possibility is afforded that, following incubation of tissues with homocysteine and formate, subsequent hydrolysis with hydriodic acid might lead to the formation of methyl iodide from sources other than methionine, additional studies will be necessary. However, these findings and those of others (20-24) suggest that folic acid may be concerned with the metabolism of 1-carbon compounds such as formic acid.

SUMMARY

The formation of labile methyl groups from formate has been demonstrated in the intact rat and in rat liver slices. This has been accomplished by administering radioactive formate and isolating and degrading methionine and choline.

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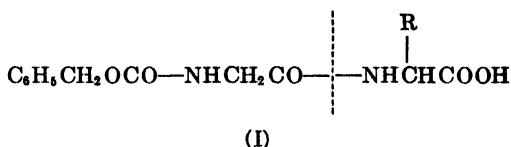
ACTION OF CARBOXYPEPTIDASE ON DERIVATIVES OF UNNATURAL AMINO ACIDS*

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Many earlier studies of the specificity of crystalline pancreatic carboxypeptidase have revealed that this enzyme can hydrolyze a great variety of acylated dipeptides, and that the rate of hydrolysis is greatly influenced by the nature of the terminal amino acid which bears the free carboxyl group (1-3). For carbobenzoxyglycylamino acids of the type shown, the variation of rate of hydrolysis is essentially a function of the nature of the side chain R. Compounds containing amino acids with aromatic R groups are hydrolyzed much more rapidly than those with aliphatic residues.



Recent studies (4-6) have shown that various carboxylic acids are inhibitors of carboxypeptidase. The efficiency of the inhibitor depends largely on the nature of the R group in much the same manner as do the substrates possessing a sensitive peptide bond (6). In addition to those inhibitors which contain residues similar to the natural amino acids, other carboxylic acids can inhibit the enzyme, *e.g.*, naphthaleneacetic acid and cyclohexanepropionic acid. Considerable evidence is at hand which indicates that the binding by the enzyme of the R groups is not due to strictly chemical forces but involves low energy short range dispersion forces, those ordinarily called van der Waals' forces (6). Such forces do not possess a high degree of specificity, such as those of purely chemical bonding, but exhibit a low specificity which is dependent on the size and nature of the attracting group.

It is evident from the foregoing that carboxypeptidase should be able to hydrolyze many compounds of the type shown in I in which the R

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groups have an energetic character and steric properties similar to those found in the natural amino acids. Preliminary studies have shown that this is indeed true (7). In this investigation, we have studied the action of crystalline carboxypeptidase on a variety of compounds containing amino acids not found in nature in order to obtain quantitative data under the same conditions as those customarily employed for the study of this enzyme.

EXPERIMENTAL

The crystalline bovine carboxypeptidase was isolated from frozen pancreas by the procedure of Anson (8) and recrystallized three times as described by Neurath, Elkins, and Kaufman (9). The final recrystallization was performed by the procedure of Smith, Brown, and Hanson (10). The enzymatic experiments were performed in 2.5 cc. volumetric flasks at 25°. The substrate solutions were buffered at pH 7.5 by the presence of 0.04 M veronal. Hydrolysis was estimated on 0.2 cc. samples by the alcoholic titration of liberated carboxyl groups (11). The preparation of carbobenzoxyglycylthienyl-DL-alanine, carbobenzoxyglycyl-*p*-tolyl-DL-alanine, and carbobenzoxyglycyl- β -naphthyl-DL-alanine is described elsewhere.¹ All of these carbobenzoxy dipeptides were used in the racemic form at 0.1 M concentration. Hydrolysis was computed on the basis of complete splitting of the L form. It has been demonstrated that carboxypeptidase does not hydrolyze compounds in which the terminal amino acid bearing the free carboxyl group is of the D configuration (1, 12).

Results

Carbobenzoxyglycyl-DL-phenylalanine (CGP) was used as the control substrate in these studies. As shown in Table I, the hydrolysis of this compound gives increasing values of *C* (proteolytic coefficient) as the action proceeds; this has previously been described and analyzed in detail by Elkins-Kaufman and Neurath (13). The *C* values found by us are in the range repeatedly observed for this compound.

The data in Table I show that the hydrolysis of carbobenzoxyglycyl-*p*-tolylalanine (previously referred to as carbobenzoxyglycyl-*p*-methylphenylalanine (7)) occurs at about one-half the rate of that of the unsubstituted compound. Stahmann, Fruton, and Bergmann (1) reported that carbobenzoxyglycyl-L-tyrosine is hydrolyzed at about half the rate of that of the corresponding phenylalanine compound. Therefore, it appears that the presence of either a methyl or a hydroxyl group in the para position has the same effect in diminishing the rate of hydrolysis of the substrate.

¹ Dunn, F. W., and Dittmer, K., *J. Biol. Chem.*, in p

The thienyl ring is regarded as being isosteric with the phenyl ring. Since the two ring structures are similar in size and in resonance energy, it is not surprising that the peptide derivatives containing thienylalanine are rapidly hydrolyzed. It is important to note that the position of the sulfur atom has no significant influence as shown by the equivalent rates

TABLE I

Hydrolysis of Carbobenzoxy Peptides Containing Unnatural Aromatic Residues

The enzyme concentration (E) is given in mg. of protein N per cc. $\times 10^4$. The parenthetical values of the average proteolytic coefficients (C) are those obtained in independent experiments. C is the first order velocity constant per mg. of protein N per cc. calculated in decimal logarithms.

Substrate	E	Time	Hydrolysis	C	C , average
		min.	per cent		
Carbobenzoxyglycyl-DL-phenylalanine	0.25	30	18	11.2	6.3 (6.0)
		60	34	12.0	
		90	50	12.8	
		120	60	13.2	
Carbobenzoxyglycyl- <i>p</i> -tolyl-DL-alanine	0.50	30	19	6.0	6.3 (6.0)
		60	34	6.0	
		90	48	6.2	
		120	61	6.8	
		150	68	6.6	
		180	74	6.4	
Carbobenzoxyglycyl- β -2-thienyl-DL-alanine	0.25	210	78	6.2	8.0 (8.6)
		60	24	8.0	
		90	35	8.4	
		120	44	8.4	
		150	50	8.0	
		180	56	8.0	
Carbobenzoxyglycyl- β -3-thienyl-DL-alanine	0.375	210	59	7.2	8.3 (8.0)
		30	18	7.7	
		60	36	8.3	
		90	46	8.0	
		120	58	8.3	
		150	68	8.8	
		180	75	8.8	
		210	78	8.3	

of hydrolysis of carbobenzoxyglycyl- β -2-thienylalanine and carbobenzoxyglycyl- β -3-thienylalanine.

Table II shows the results obtained on the hydrolysis of the carbobenzoxyglycyl-naphthylalanines. Since the enzymatic action is much slower than that found with the thienyl compounds, much higher enzyme concentrations were employed. In an earlier report (7) only insignificant hy-

drolysis was found; this was obviously due to the low enzyme concentrations which were used.

Because the β -1-naphthylalanine peptide derivative was only slightly soluble in water, kinetic studies were made in the presence of methanol. It was possible to demonstrate the susceptibility of this compound to the action of carboxypeptidase but decreasing velocity constants were observed. This is in contrast to the action of the enzyme on carbobenzoxyglycyl- β -2-naphthylalanine with which satisfactory first order coefficients were obtained both in the aqueous solution and in methanol.

TABLE II

Action of Carboxypeptidase on Compounds Containing Naphthylalanine

The enzyme concentration (E) is given in mg. of protein N per cc. $\times 10^3$.

Substrate	Solvent	E	Time	Hydrolysis	C
			min.	per cent	
Carbobenzoxyglycyl- β -1-naphthyl-DL-alanine	32% methanol	41.7	30	22	0.086
			45	27*	0.072
			60	31	0.065
			90	35	0.050
			120	37	0.041
Carbobenzoxyglycyl- β -2-naphthyl-DL-alanine	32% "	12.5	30	36	0.51
			45	46*	0.47
			60	61	0.54
			90	69	0.45
Carbobenzoxyglycyl- β -2-naphthyl-DL-alanine	Aqueous	2.9	30	20	1.10
			60	34	1.03
			90	47	1.07

* A flocculent precipitate of the liberated amino acid began to form at this point.

Since the presence of methanol causes a decrease in the proteolytic coefficient of more than 50 per cent, the effect of methanol on the hydrolysis of the more sensitive substrates CGP and carbobenzoxyglycyl-L-tryptophan (CGT) was also studied. The data are shown in Fig. 1. With CGP the apparent first order proteolytic coefficient decreases with increasing methanol concentration, and the coefficients actually became more constant as the methanol concentration is increased. At 0.05 M the hydrolysis of CGT follows the kinetics of a zero order reaction to about 80 per cent of hydrolysis (2). The activity in water has been arbitrarily made equal to that of CGP. Methanol has no effect on the zero order character of the hydrolysis of CGT. Moreover, our data do not show any inactivation of the enzyme during the time that our observations were

made. It is quite clear from the data in Fig. 1 that the effect of methanol in reducing the rate of hydrolysis is similar for the three substrates.³

In regard to the question of the decreasing velocity constants observed for the hydrolysis of carbobenzoxyglycyl- β -1-naphthylalanine, the obvious interpretation is that a progressive inhibition is being produced by the reaction product, β -1-naphthyl-L-alanine. It is apparent that carbobenzoxyglycine does not inhibit this enzyme, since it is produced during the hydrolysis of the other substrates. Indeed, it has also been directly demonstrated that this substance does not inhibit carboxypeptidase (4). Preliminary tests showed that the hydrolysis of carbobenzoxyglycylphenyl-

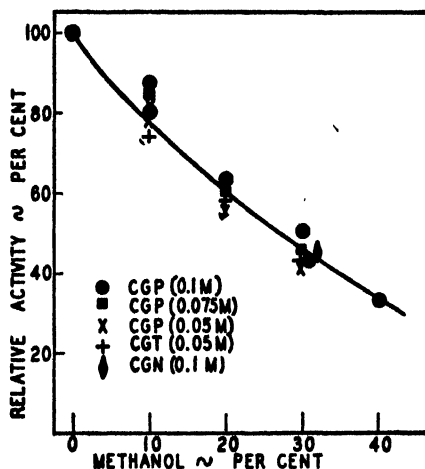


Fig. 1. Effect of methanol on the action of carboxypeptidase. The values in aqueous solution for carbobenzoxyglycyl-DL-phenylalanine (CGP), carbobenzoxyglycyl-L-tryptophan (CGT), and carbobenzoxyglycyl- β -2-naphthyl-DL-alanine (CGN) have been arbitrarily called 100 per cent. It is apparent that the effect of methanol is the same for the action on the three substrates.

alanine is inhibited by β -1-naphthyl-DL-alanine in a competitive manner; i.e., inhibition increases as the substrate is hydrolyzed. However, until the optically active naphthylalanines are available, no definite conclusions can be drawn from this, particularly since Elkins-Kaufman and Neurath (4) have shown that D-amino acids are much more effective inhibitors of carboxypeptidase than the L isomers.

³ More detailed studies of the effect of methanol in diminishing the hydrolytic action of carboxypeptidase have been made by Dr. R. Lumry of this laboratory. Methanol appears to act as an uncompetitive inhibitor; i.e., its action is similar to a diminution in enzyme concentration.

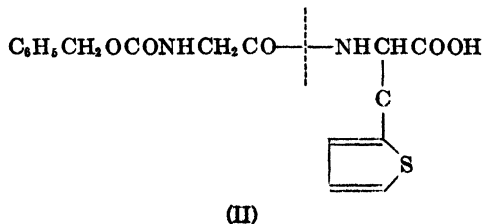
Extrapolation indicates that the initial proteolytic coefficient for carbobenzoxyglycyl- β -1-naphthylalanine is 0.11 in 32 per cent methanol. With the average change in C for the different substrates shown in Fig. 1, 32 per cent methanol causes a diminution in rate of 57 per cent. The proteolytic coefficient of the β -1-naphthylalanine derivative is calculated to be 0.26 in water, which is about one-fourth of the C value for the corresponding β -2-naphthylalanine derivative.

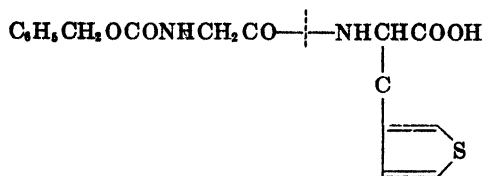
DISCUSSION

The ability of carboxypeptidase to hydrolyze compounds which contain unnatural amino acids opens a vast field of investigation, since it now becomes possible to undertake a systematic investigation of the nature of the interaction of the side chain of the terminal amino acid with the protein. Although such an investigation is now in progress in this laboratory and definitive discussion should be postponed until such studies are complete, it may be desirable to point to a few conclusions which are already apparent.

It has previously been indicated that the binding of the side chains of the substrates of certain peptidases must be due to van der Waals' forces (4, 6, 14, 15). For leucine aminopeptidase, these side chains are aliphatic residues which cannot be expected to interact with primary valences. The variation in rates of hydrolysis of the aliphatic amino acid amides can be explained by invoking van der Waals' interaction with the protein (15).

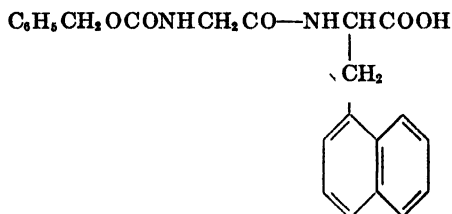
The wide specificity of carboxypeptidase towards substrates containing the natural amino acids likewise indicates a binding to the enzyme which does not involve primary bonds. This is certainly substantiated both by the inhibition of this enzyme produced by acids which contain residues not related to the naturally occurring amino acids (6), and by the hydrolysis of substrates of appropriate structure which contain such residues. It is particularly striking that carbobenzoxyglycyl- β -2-thienylalanine (II) is hydrolyzed at the same rate as carbobenzoxyglycyl- β -3-thienylalanine (III). Since the isomeric thienyl rings are chemically different but interact with carboxypeptidase to the same extent, we can only infer that this interaction depends on the total energy of interaction of the entire ring and not on any particular part of it.



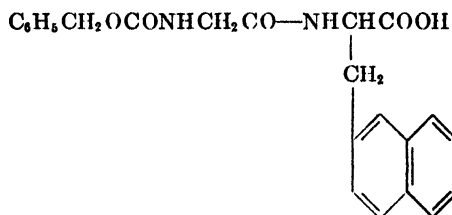


(III)

On the other hand, the difference in carboxypeptidase interaction with carbobenzoxyglycyl- β -1-naphthylalanine (IV) and carbobenzoxyglycyl- β -2-naphthylalanine (V) may be due to a steric effect or to the energetic character of the naphthalene rings; it is well known that 1-naphthyl derivatives differ in chemical behavior from 2-naphthyl derivatives.



(IV)



(V)

SUMMARY

1. Crystalline pancreatic carboxypeptidase can hydrolyze carbobenzoxy dipeptides which contain a terminal amino acid of a character not found in nature.

2. Carbobenzoxyglycyl- β -2-thienylalanine is hydrolyzed at the same rate as carbobenzoxyglycyl- β -3-thienylalanine; this action is at about two-thirds the rate of hydrolysis of the corresponding phenylalanine derivative.

3. Carbobenzoxyglycyl- β -2-naphthylalanine is hydrolyzed about 4 times more rapidly than the isomeric β -1-naphthylalanine derivative. The hydrolysis of the latter compound is subject to a progressive inhibition apparently caused by the liberation of the split-product, β -1-naphthylalanine.

4. Carbobenzoxyglycyl-*p*-tolylalanine is hydrolyzed at the same rate as the corresponding hydroxyl compound, carbobenzoxyglycyl-L-tyrosine.

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FURTHER STUDIES ON THE OXYGEN EQUILIBRIUM OF HEMOGLOBIN

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The heme proteins constitute perhaps the most varied and beautiful class of molecular machines known to the biochemist. Whether we think of the cytochromes, acting as essential links in the main chain of biological oxidations, or the peroxidases and catalases, or the respiratory pigments, these compounds may all be regarded as enzymes. This point of view is perhaps somewhat unexpected when applied to the respiratory pigments, which are more commonly described as transporting mechanisms for oxygen than as enzymes, but it is, none the less, justified if we think of oxygen as a substrate which is taken on and later liberated unaltered in the region where it is being called for. Oxygen has in the meantime been bound to the enzyme in a profoundly modified state, for magnetic studies show that the oxygen molecule, while in combination with hemoglobin, is transformed from a paramagnetic to a diamagnetic configuration. Between the diverse compounds of heme and protein there are great differences in the nature of the molecular machine, which reflect differences of function. Clearly it is of the greatest interest to develop as complete a picture as possible of each type of molecule and to present this in terms of differences of structure. Some progress has already been achieved as a result of the work of many investigators. The present paper deals with the problem only in respect to one group of heme proteins, namely the respiratory pigments. It is directed, quite specifically, to the task of providing further elucidation of the nature of the attachment of the heme to the globin and to the interaction which occurs between the hemes, or at least between the oxygen-combining centers, whenever more than one heme is present in the molecule.

Much information on the structure of mammalian hemoglobin has resulted in the last 10 years from the x-ray and optical studies of Perutz and his associates on single crystals (1, 6). It is now fairly clear that in the case of oxyhemoglobin of the horse (and some other hemoglobins as well) the molecules are essentially cylindrical or prismatic in shape, being approximately 34 Å high and 57 Å across. There is a 2-fold axis of symmetry perpendicular to the cylinder (or prism) axis, and the four hemes are arranged with their planes parallel to these two axes. There is some evidence from the same source that the hemes occur in pairs. These

conclusions are in good agreement with inferences based on quite a different set of considerations involving the physicochemical behavior of hemoglobin. Of particular significance are the equilibria between oxygen, protons, and hemoglobin and their dependence on temperature, as well as oxidation-reduction equilibria, and also the way in which all these equilibria are affected when the molecule is split in strong urea solutions. An account of much of this has been developed at some length by one of the present authors in a recent review (7), in which the fundamental theoretical relations, as well as the experimental facts involved, have been set forth in detail.

The four protohemes of vertebrate hemoglobin are all identical, and it is established beyond reasonable doubt that they represent the four points of attachment of oxygen. Whether or not, however, these four sites are identical in their behavior towards oxygen must depend not only on the hemes themselves but also on the groups of the globin to which they are bound. Now the fact emerges from the observations of a number of investigators that when Y , the fractional saturation of hemoglobin with oxygen, is plotted against $\log p$, p denoting the partial pressure of oxygen, the resulting curve is invariant in *shape* for changes of temperature and of pH. The same invariance of *shape* with changing pH is also displayed by the curves representing the oxidation-reduction equilibrium. If strictly true, this is a very fundamental point, for it may be shown to imply that the number of protons dissociated and the amount of heat liberated as a result of combination of hemoglobin with oxygen are the same for each successive molecule of oxygen which combines. (The same is true for each step in the four step oxidation process.) This could hardly be interpreted otherwise than as meaning that the four oxygen-combining centers are all equivalent and consequently that the heme-globin linkage is the same for each; this conclusion has been widely accepted as a basis for further rationalizations. From a more detailed study of the effect of temperature on the oxygen equilibrium, more specifically from its effect on the pH dependence of the oxygen equilibrium, it has been possible to estimate the heat of dissociation of the two acid groups which are known to interact with each oxygen-combining group as about 6500 calories. This has led to the suggestion that these groups are both imidazole groups of histidine and, together with observations on the magnetochemical properties of hemoglobin and oxyhemoglobin, has provided the basis of an electronic picture of the heme-globin linkage (7). Moreover, the character of the oxygen and oxidation-reduction equilibria of hemoglobin, which has been split in urea solutions, implies that the two parts of the split molecule are identical (at least in regard to these func-

tions) and that each contains a pair of strongly interacting hemes.¹ So we are led to believe that, unless there is a very considerable reconstitution of the molecule accompanying splitting, native hemoglobin contains two identical pairs of strongly interacting hemes. The steepness of the curve of Y versus $\log p$ at its mid-point (related to the n of the approximately applicable equation $Y = (Kp^n)/(1 + Kp^n)$ in which K is a constant) implies further but weaker stabilizing interactions between hemes of different pairs. It seems fairly clear, on the basis of the increase in the absolute oxygen affinity of hemoglobin, which results from splitting the molecule, that these weaker interactions stem from the unoxygenated rather than the oxygenated hemes.

Thus, some progress and some degree of consistency have been achieved in our efforts to provide a blue-print of the functioning hemoglobin molecule. But, although the theoretical foundations of many of the conclusions just outlined are rigorous, the validity of the picture is limited by the accuracy of the observations, many of which, particularly those involving second order effects, are subject to a considerable error. It is desirable, therefore, to reexamine some of the crucial facts with more accurate methods. For instance, is it really true that the oxygen equilibrium curve (Y versus $\log p$) is invariant in shape for changes of pH? Or, will more exact measurements bear out our value for the heat of dissociation of the oxygen- (heme) linked acid groups? Again, we should like more extensive and more exact observations on the Bohr effect; that is, the interdependence of the oxygen and acid-base equilibria. Moreover, it is certainly important to extend observations of this kind to other hemoglobins, not only those of mammals, but also those of other vertebrates and invertebrates, in which the molecular weights and oxygen affinities may be very different.

In addition to this there is a perplexing phenomenon which, though it involves a rather minor difference as far as the measurements are concerned, is of great theoretical importance in relation to the configuration of the hemoglobin molecule. This deals with the symmetry of the curves in which Y is plotted against $\log p$. We shall show later that, if the four hemes are so arranged that they are all equivalent, not only in virtue of their identity and the identity of their linkages to the globin, but also in virtue of their interactions with one another, then they must be expected to give rise to a symmetrical oxygen equilibrium curve, and conversely that a symmetrical equilibrium curve demands that the interacting

¹ In this discussion the term "heme" is at times used somewhat loosely to denote the reactive group constituted by the heme as combined with the globin.

hemes all be equivalent to one another. The proof presupposes only that the free energies of interaction of the hemes are additive.³ If the curve were symmetrical with the strong interactions which we know to prevail, but the heme-globin configurations were not equivalent, then we should have to postulate an almost inconceivable adjustment of the interactions precisely such as to compensate for the inherent differences among the four configurations. Now what appear to be the most accurate and reliable of the earlier data on mammalian hemoglobin (the results of Forbes and Roughton (4) and of Courtice and Douglas (2)) indicate very significant departures from symmetry in the curves for human and sheep hemoglobin. This would appear to be at variance with the results of Perutz, based on x-ray and optical phenomena, and unexpected in view of the inferred equivalence of the four oxygen-combining centers indicated by the physicochemical data, although, as a second order effect, too much weight should not be attached to this last point. The results might of course be ascribed to small errors in the experiments or to modifications in the hemoglobin as studied in the oxygen equilibrium experiments. It is known that hemoglobin is a delicate molecule easily altered by even mild treatment. But in any case this whole matter of the symmetry clearly points to the need of more searching experiments and offers added incentive to the development of a new, easier, and more accurate experimental method than any now available for determining oxygen equilibrium curves. Such a method, a photometric one, has now been worked out.

In the development of a method the prime requirement is accuracy. At the same time, since hemoglobin in solution is known to be subject to change, speed is an important consideration. The measurements should be made as quickly and with as little manipulation as possible in order to minimize irreversible alteration of the hemoglobin in the course of the experiment. This points to a spectrophotometric procedure. Another consideration is that the method should be applicable to as concentrated solutions as possible, since proteins are in general more stable at high concentrations and, in particular, many hemoglobins are known to dissociate at low concentrations. This suggests the need of working with light of a wave-length for which hemoglobin is fairly transparent, as well as with one for which there is a considerable difference in absorption between the oxygenated and deoxygenated forms of the pigment. Another

³ This means that if we are considering three hemes, designated by 1, 2, and 3, and if the free energy of oxygenation of 1 is ΔF_1 when 2 and 3 are unoxxygenated, but $\Delta F_1 + \Delta_2 F_2$ when 2 is oxygenated but 3 is not, and $\Delta F_1 + \Delta_1 F_1$ when 3 is oxygenated but 2 is not, then the free energy change is $\Delta F_1 + \Delta_2 F_2 + \Delta_1 F_1$ when both 2 and 3 are oxygenated.

thing to be considered is the amount of solution required for the measurements. If the method is to be applicable to some of the rarer hemoglobins (or erythrocruorins), such as those from small invertebrates or from the root nodules of leguminous plants, it should require only the smallest possible amount of material. The method which we have worked out appears to meet these various demands fairly satisfactorily. It avoids the necessity of making any gasometric measurements at all, it involves no significant corrections for dissolved oxygen, it is applicable to solutions of the highest attainable concentrations, and it demands the use of only 1 cc. or less of solution for a complete experiment.

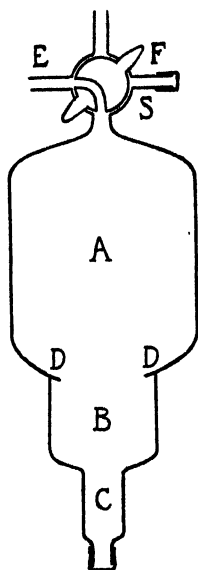


FIG. 1. Tonometer. *A*, *B*, and *C*, chambers; *D* glass collar; *E* and *F* outlet tubes; *S*, stop-cock.

The principle of the method is to be found in a specially designed tonometer shown in Fig. 1. This is of course subject to considerable variation to suit the particular problem in hand. The present description is based on a model that has been used in a large number of experiments. The total volume of the vessel (chamber *A* + *B* + *C*) is about 300 cc. Chamber *C* is an accurately made optical cell having a thickness (path length of the light through the solution) of 2.5 mm. It opens below into a nipple which can be sealed by a rubber vaccine cap and above into chamber *B* having a volume of about 50 cc. 1 cc. of liquid is required to fill chamber *C* and the nipple. Filling may easily be accomplished through the vaccine cap by means of a syringe and fine hypo-

dermic needle, or it may be done before the cap is applied to the nipple. Exacting tests show that when a needle is withdrawn from the cap the rubber closes up instantly and remains gas-tight. Chamber *A* communicates to the outside by one or other of two pathways (tubes *E* and *F*) via stop-cock *S*. One of these (*F*) is closed by a second vaccine cap; the other is used for evacuation and related operations.

When the protein solution is to be equilibrated with a gas mixture, the whole tonometer is rotated with its long axis horizontal in a constant temperature bath. During this process the solution flows down into chamber *B*, but is prevented by the glass collar *D* from entering the larger chamber *A*, where too much would be lost as a result of spreading over the walls. After each equilibration, the tonometer is removed from the bath for spectrophotometric analysis (in an upright position), as will be described presently. The course of the equilibrations is as follows: The oxyhemoglobin solution is introduced into *C* (and *B*). The air in the tonometer is then replaced by oxygen-free nitrogen by means of successive partial evacuations and admissions of nitrogen through tube *E*, equilibrations being carried out during this process. It is found to be important in doing this not to let the solution "boil," owing either to evaporation of water or sudden extraction of oxygen, as the foaming leads to considerable denaturation of the protein. In order to remove oxygen from the other exit tube *F*, which is closed by a vaccine cap, stop-cock *S* is turned back and forth during this process. When all the oxygen has been removed from the system (including of course the protein) in this way, the experiment may be begun. Assurance of complete removal of oxygen may be obtained from a constancy in the absorption coefficient of the solution. This whole initial procedure requires nearly an hour. Slight loss of water vapor and consequent concentration of the protein which may occur in this time are not important, since the spectrophotometric analysis for the points on the oxygen equilibrium curve is based entirely on subsequent measurements. During all measurements, and indeed at all times subsequent to the last evacuation, stop-cock *S* is left in such a position as to connect *A* with outlet *F*, which is closed by a vaccine cap.

As soon as the final absorption measurement on the completely deoxygenated solution is complete, a desired amount of air is introduced into the tonometer by means of a syringe and hypodermic needle, calibrated as to volume of delivery, through the vaccine cap closing tube *F*. For the greatest accuracy the syringe is mounted in a special holder and the movement of the piston is controlled by a stop. The resulting pressure of oxygen in the tonometer is obtained very simply from the known composition of air and the ratio of the gas phase in the tonometer to the volume of air introduced. In making this calculation it is necessary to take

account of the barometric pressure and the water content of the air, the latter being determined by means of a wet bulb thermometer. It is also necessary to make a correction for the amount of oxygen taken up by the solution. This is based on the percentage saturation of the hemoglobin with oxygen as given by the photometric measurements and on the concentration of the hemoglobin. This correction is usually small, amounting to about 5 per cent of the calculated pressure of oxygen in experiments in which the concentration of protein is 10 per cent, but it depends, of course, on the oxygen affinity of the hemoglobin being studied. It can, of course, be determined with high accuracy if that is desired. The uncertainty in the corrected values of p may be estimated as 1 per cent or less.

Attainment of equilibrium after each admission of air requires rotation for about 10 to 15 minutes. The last measurement is made on essentially 100 per cent oxygenated hemoglobin obtained by exposing the solution to atmospheric air. If it were necessary, pure oxygen could of course be used at this point.

The fraction of the hemoglobin in the oxygenated form resulting from any particular equilibration is calculated from the measured value of the absorption coefficient in relation to the two basic values, that of the deoxygenated solution obtained at the start of the experiment and that of the completely oxygenated solution obtained at the end of it. In making the calculations the applicability of Beer's law is assumed. This is completely justified by measurements on a series of oxyhemoglobin solutions of known dilutions and by the fact that the results obtained in this way are in complete agreement with those obtained under similar conditions by gasometric methods. The applicability of Beer's law means, of course, that the light used in the analysis is sufficiently monochromatic so that its spectral composition does not change significantly as it passes through the solution. It should be noted that no knowledge of the absorption coefficients themselves is required in these calculations.

The spectrophotometric procedure consists in measuring the intensity of a beam of red light ($\lambda \cong 700 \text{ m}\mu$ or slightly greater) before and after its passage through the absorption cell. This wave-length was chosen as satisfying our basic requirement that it should be readily transmitted by both oxygenated and deoxygenated hemoglobin, although much more readily by one than the other. For $\lambda = 706 \text{ m}\mu$ the absorption coefficient as given by Horecker (5) is 390×10^3 sq. cm. per gm. atom of iron for hemoglobin and 98×10^3 for oxyhemoglobin. In making the measurements we have made use at times of a simple vacuum type photoelectric tube (RCA 919) and at other times of a photomultiplier tube (RCA 931). The current produced by the light falling on the tube is passed through a

suitably chosen high grade resistor of low "noise level" and the resulting voltage drop is measured by means of a potentiometer and detector for high resistance circuits. In certain of the measurements this was a special bridge involving an FP-54 tube which was originally developed for glass electrode work and in others a Millivac vacuum tube voltmeter made by the Millivac Instrument Company. By using the photomultiplier tube, it proved convenient to have several resistors of different values ranging from 0.1 to 2 megaoohms which could be selected by means of a switch in accordance with the intensity of the light to be measured. Voltage measurements were easily made to a fraction of a millivolt and, since the signal was usually of the order of a large fraction of a volt, if not more than a volt, there was no significant error involved in measuring it. It was, however, necessary to make a correction for the dark current from the cell, and, since this showed some drift with time, it had to be determined at frequent intervals. In the case of the photomultiplier tube (whose maximum sensitivity was far from the wave-length used) this correction amounted to about 3 per cent of the average signal; in the case of the simpler 919 tube the correction amounted to about 8 per cent of the average signal. Since it could easily be determined to about 3 per cent of its own value, it was not a serious source of error.

Basically each measurement consisted first of a determination of I_0 , the intensity of the incident light, then one of I , the intensity of light emerging from the cell, and finally one more measurement of I_0 . The alternation could, of course, be continued if it was found that there was a significant drift in I_0 during the course of a measurement. In order to minimize errors due to drifts and fluctuations of I_0 it was important to have a very constant light source. That which was used was a simple 6 volt, 30 watt microscope lamp run from two heavy duty accumulators connected in parallel. These were given a trickle charge about equal to the current being drawn from them by the lamp during a measurement. Under these conditions the value of I_0 was generally constant to about 1 per cent over a period of several minutes. A single measurement took but 2 minutes. Essentially monochromatic light of wave-length ~ 700 μ was obtained by means of an interference filter. In one set of experiments a Farrand filter having a peak transmission at $\lambda = 695$ μ was used; in another a Baird filter backed by a masking filter of Corning glass (glass code No. 2403, color specificity No. 2-58, H. R. pyrometer shade red) which gave maximum transmission at $\lambda = 707$ μ . It was also convenient to have a calibrated neutral filter made from a piece of photographic film, which could be introduced at will to reduce the value of the light in measurements of I_0 .

Having regard to all sources of error, we may estimate the accuracy

in the measurement of I/I_0 as about 0.5 per cent. As we have pointed out, errors in p , the partial pressure of oxygen in the tonometer, are of the order of 1 per cent. Both are small. The principal errors in our results are probably to be ascribed to progressive changes in the hemoglobin during the course of the measurements, incomplete deoxygenation before the beginning of the experiment, and the fact that the solution may from the start have contained small but significant amounts of degeneration products, which take part in the oxygen equilibrium. Very small deviations from Beer's law may have had a just appreciable effect.

The first experiments, designed to provide an empirical check of the over-all reliability of the method, were made on solutions of recrystallized human hemoglobin prepared from cells obtained from the Biologic Laboratories of the Massachusetts State Department of Public Health. They were all highly consistent with one another and with the experiments of Courtice and Douglas (2), made with great precautions by their gasometric method on whole blood. Fig. 2 shows the results of a typical experiment, together with all the results of Courtice and Douglas, for comparison. In this figure Y , the fractional saturation of the hemoglobin with oxygen, is plotted against $\log p$. Our own measurements were made on a 9.4 per cent³ solution in a 0.08 M phosphate buffer of pH 7.17 at 20°. The results of Courtice and Douglas were made on unbuffered whole blood subjected to a partial pressure of carbon dioxide of 40 mm. and at 37°. They involve the blood of three different individuals, distinguished by different symbols. In plotting these results of Courtice and Douglas we have applied a small correction to take account of the progressive drop of pH accompanying oxygenation. This was based on the Bohr effect as it is observed in horse hemoglobin. The three blood samples showed small differences in the absolute value of their oxygen affinity, and in the figure the points for each have been superposed to best advantage on our own results by displacement along the $\log p$ axis. For this reason absolute values of $\log p$ in Fig. 2 are without significance. It will be seen that the agreement in regard to the shape of the curve between these results and our own is very close and certainly lies within

³ In all these experiments protein concentrations were calculated from the difference between the absorption ($\log I_0/I$) of hemoglobin and that of oxyhemoglobin on the basis of Hgrecer's (5) values of the absorption coefficients. The results obtained in this way agreed with the values calculated from the oxygen capacity of the solutions to 10 per cent or better. Any discrepancy may be attributed to errors in the oxygen capacity measurements, and, to a larger extent, to uncertainty as to the effective wave-length of the light used in the optical measurements and the correct values of the absorption coefficients. Optical measurements made before and after the completion of an experiment indicated that in almost all cases no more and usually less than 2 per cent of the protein was inactivated as a result of the manipulations.

the scatter of the Courtice and Douglas points. There can be little doubt, therefore, of the validity of the new method, which yields at least somewhat more consistent, if not indeed more accurate, results than the gasometric methods and with far less trouble.

It should perhaps be pointed out here that the results of Forbes and Roughton (4) on solutions of defibrinated and laked but unpurified sheep blood, diluted thirty to forty-five times with borate buffers ranging from pH 7.8 to 9.3, are all essentially identical (regarding shape of the curve) with those contained in Fig. 2. This is the more striking when it is re-

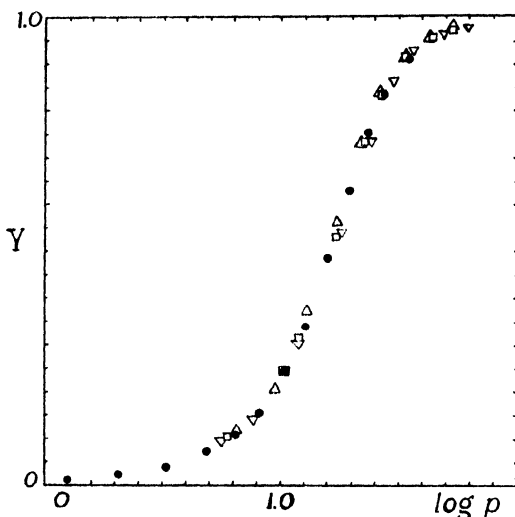


FIG. 2. Comparison of authors' results on solutions of human hemoglobin with results of Courtice and Douglas on whole human blood. Y denotes fractional saturation of hemoglobin with oxygen; p denotes partial pressure of oxygen. Results of different experiments have been superposed by suitable displacements along $\log p$ axis. $\bullet$, authors' points; Δ , $\square$, ∇ , points from Courtice and Douglas on blood from T. J. M., C. G. H., and F. C. C., respectively.

called that the globins of sheep and human hemoglobin are certainly different both in respect to amino acid composition and structure of the crystal lattice, as revealed by recent work of Perutz and his associates.

It would appear, therefore, that the new method confirms the earlier results regarding the unexpected asymmetry of the oxygen equilibrium curves. Quite a different picture was obtained, however, when the measurements were made on much fresher preparations of hemoglobin. The cells obtained from the Biologic Laboratories were several days old by the time of laking; that is, several days had elapsed since the original drawing of the blood from the donor, and it was a matter of at least several days

more before the actual experiments were made. In view of this it seemed desirable to carry out measurements with our method, whose validity had now been tested, on solutions of freshly prepared hemoglobin. Studies were made, therefore, on hemoglobin from blood freshly drawn from the experimenter. A few cc. were enough. This blood was washed four times with 0.9 per cent NaCl solution and laked by mixing with an equal volume of water and 0.4 volume of toluene, and allowing it to stand for $1\frac{1}{2}$ hours. The stroma was removed by centrifuging and the hemoglobin solution pipetted off. The solution was then buffered with phosphate of the desired pH and after a final centrifugation was ready for use. All operations were performed in a cold room at $2-3^{\circ}$, and all solu-

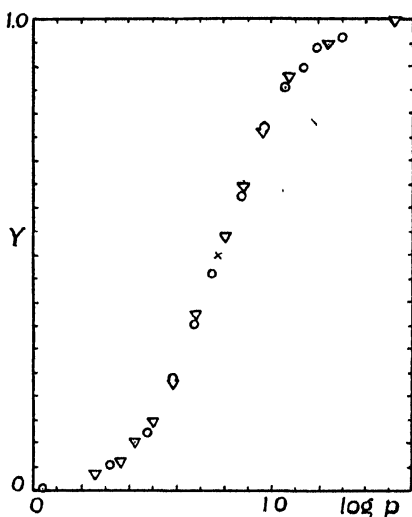


FIG. 3. Oxygen equilibrium data on freshly prepared human hemoglobin at pH 7.49. O, original points; Δ , points obtained by rotating the figure 180° about the point corresponding to $Y = 0.5$, indicated by X, to show symmetry.

tions were chilled before addition to the protein. The measurements were begun about 5 hours after the blood was drawn and completed 12 hours later. They were all made at 20° .

Fig. 3 shows the results obtained in a typical experiment on such freshly prepared hemoglobin. In this particular experiment the protein was at a concentration of 17.6 per cent in a 0.4 M phosphate buffer of pH 7.49. The fundamental symmetry of these data is brought out by the two sets of points, which give the actual experimental points and the positions of these points when Fig. 3 is rotated in its own plane 180° around the center of the curve, for which $\log p_1 = 0.78$.

Similar results were obtained with other samples of fresh human blood

from other individuals.. The curves were for the most part symmetrical, like those shown in Fig. 3, though in some experiments there were small departures from symmetry in the direction of the results of Courtice and Douglas. The other samples studied included maternal blood drawn at the time of childbirth and fetal blood investigated in the course of a comparative study of fetal and adult blood being carried out in collaboration with Dr. Clement Smith of the Boston Lying-in Hospital, the results of which will be reported in a later paper. For all these cases the value of

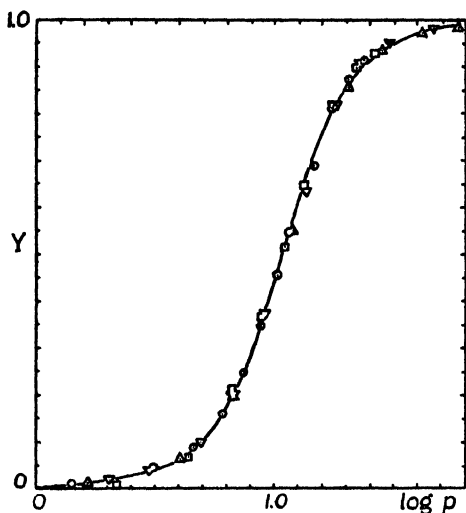


FIG. 4. Oxygen equilibrium data. ∇ , hemoglobin from D. W. A. at a concentration of 18.1 per cent in 0.4 M phosphate at pH 7.49. Δ , hemoglobin from K. F. G. at a concentration of 7.4 per cent in borate buffer at pH 8.87 and ionic strength 0.6. $\square$, maternal hemoglobin at a concentration of 2.8 per cent in 0.1 M phosphate at pH 7.4. $\circ$, term fetal hemoglobin at a concentration of 19.2 per cent in 0.4 M phosphate at pH 7.45. Results of different experiments have been superposed by suitable displacement along $\log p$ axis. The smooth curve is the theoretical one described in the text.

n was 2.90 ± 0.05 , the same as the value given by earlier studies on horse and sheep hemoglobin. All this is brought out by Fig. 4, which shows for comparison points obtained on hemoglobin from two of the present authors (K. F. G. and D. W. A.) and on maternal and term fetal hemoglobin from the Boston Lying-in Hospital.⁴ The smooth curve in Fig. 4, which

⁴ Whenever curves for a given type of hemoglobin have been displaced to show identity of shape with other curves for the same type, the displacements correspond closely with predictions based on the Bohr effect. Whether or not fetal and maternal human hemoglobins are identical in respect to the absolute values of their oxygen affinities is not proved, but any difference is certainly small.

is symmetrical, is a theoretical one calculated on the basis of a model discussed previously (7), with $\alpha = 360$ and $\beta = 3.6$.

It is of considerable interest to observe that other experiments made by Mr. Austin Riggs with the same method on hemoglobin from bullfrog blood give the same symmetrical curve, with n also equal to about 2.9, as that obtained with human hemoglobin. In the case of bullfrog tadpoles the results at pH 7.3 were essentially the same ($n \cong 2.8$, curves symmetrical). At higher and lower pH values, the value of n is less, presumably indicating some oxidation or splitting of the molecules. In view of the essential identity (in regard to shape) of the oxygen equilibrium curves of bullfrog and tadpole hemoglobins, it is striking, indeed, that these results of Mr. Riggs show that, whereas the Bohr effect in bullfrog hemoglobin is, qualitatively at least, like that in human and other mammalian hemoglobins, there is no Bohr effect at all in tadpole hemoglobin. The results will be presented in a later paper.

It may not be out of order to remark here that our results on the oxygen equilibrium of fresh preparations of human hemoglobin are duplicated by recent results of Roughton and Paul (8) on more concentrated solutions of sheep hemoglobin than those previously studied in the work of Forbes and Roughton. It would seem, therefore, with regard to all the facts, that symmetry is after all a very fundamental feature of the equilibrium curves of oxygen and hemoglobin.

As was pointed out earlier, one of the objectives of developing our new method was to investigate the question of the invariance of shape of the equilibrium curve (Y versus $\log p$) with pH. Several curves were made on fresh preparations of human hemoglobin at different pH values with this question in view. Fig. 5 shows the three sets of points obtained, respectively, at pH 6.43, 7.49, and 8.87, all superposed to test the invariance of the curves. The full curve is symmetrical. It will be seen that the results for pH 7.49 and 8.87 are indistinguishable. The points for pH 6.43 lie slightly above the other two sets of points in the lower range and are in fact somewhere between the symmetrical curve obtained with fresh preparations of hemoglobin at higher pH values and the original asymmetrical curve obtained with older preparations,

In regard to the question of the effect of pH on the shape of the oxygen equilibrium curve, we believe the curve to be invariant with pH. The identity of shape (to within the experimental error) of the curves obtained at pH 7.49 and 8.87 bears this out completely. The small difference between these curves and the curve at pH 6.43 does not appear to be significant. It is known that in mildly acid solution hemoglobin has a tendency to be spontaneously oxidized in the presence of molecular oxygen. It is also to be expected that a partial oxidation (inactivation of oxygen-combining power) of hemoglobin will destroy the symmetry of the equi-

librium curve, and, with the assumption that the stabilizing interactions between oxygen-combining centers have their origin in the unoxxygenated hemes, it is further to be expected that the base of the curve will be shifted to the left more than the upper part. These expectations are in the main substantiated by studies on the oxygen equilibrium of mixtures of hemoglobin and oxidized (ferri-) hemoglobin of Darling and Roughton (3). In accordance with the argument referred to in this section we feel justified therefore in our conclusion that all four heme-globin configurations are the same, which was strongly indicated by all the earlier experiments.

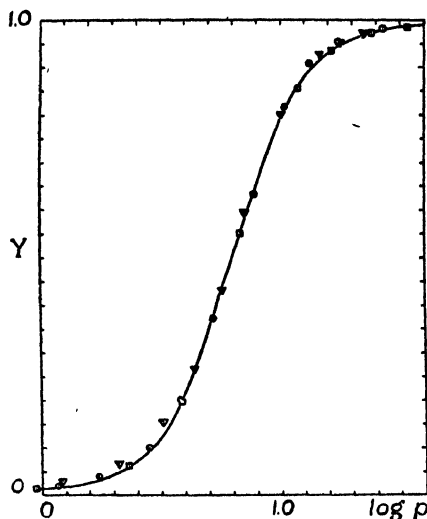


FIG. 5. Oxygen equilibrium data to show invariance of shape of equilibrium curve with pH. Absolute values of abscissae are without significance in this figure. O, hemoglobin from D. W. A. at a concentration of 18.1 per cent in 0.4 M phosphate at pH 7.49. $\log p_1 = 0.820$, when p_1 is the pressure for half saturation. □, hemoglobin from K. F. G. at a concentration of 7.4 per cent in borate buffer at ionic strength 0.6 and pH 8.87. $\log p_1 = 0.324$. Hemoglobin from D. W. A. at a concentration of 17.6 per cent in 0.4 M phosphate at pH 6.43. $\log p_1 = 1.116$.

This important conclusion appears to be in accord with recent unpublished experiments of Roughton in which he has studied the extreme upper portion of the equilibrium curve of carbon monoxide and hemoglobin by an ingenious gasometric method. Carbon monoxide is known to combine with hemoglobin at exactly the same sites as oxygen, and the equilibrium curves (Y versus $\log p$) for the two reactions are of the same shape. From his results Roughton is able to obtain very simply the last of the over-all constants for the equilibrium. If we write as the most general formulation of this equilibrium the equation

$$(1) \quad Y = \frac{L_1 p + 2L_2 p^2 + 3L_3 p^3 + 4L_4 p^4}{4(1 + L_1 p + L_2 p^2 + L_3 p^3 + L_4 p^4)}$$

this constant is equal to L_4/L_3 . Now Roughton's results appear to show that the percentage variation of this constant with both pH and temperature is the same as that which we calculate for the over-all carbon monoxide affinity of hemoglobin; *i.e.*, the reciprocal of the partial pressure of the gas required to half saturate the protein.

$$(2) \quad \left(\frac{\partial \log L_4/L_3}{\partial \text{pH}} \right)_T = - \left(\frac{\partial \log p_{\frac{1}{2}}}{\partial \text{pH}} \right)_T$$

$$\left(\frac{\partial \log L_4/L_3}{\partial T} \right)_{\text{pH}} = - \left(\frac{\partial \log p_{\frac{1}{2}}}{\partial T} \right)_{\text{pH}}$$

These two relations are, however, part of the necessary and sufficient conditions for the invariance of shape of the equilibrium curve with pH and temperature, the other relations involving L_1 , L_2/L_1 , and L_3/L_2 . The proof of this follows directly from Equations 13 and 15 of Wyman (7) already referred to.

Let us now turn to the question of the symmetry of the equilibrium curves. It follows from Equations 17 that there is just one condition for symmetry in the case of hemoglobin, in which there are only four centers for combination with oxygen (or carbon monoxide). This, in terms of the constants of Equation 1, is the following:

$$(3) \quad L_1 L_4^{\frac{1}{2}} = L_3$$

It is both necessary and sufficient. Now let us assume that the four oxygen-combining centers, which we shall distinguish by subscripts 1, 2, 3, and 4, are all inherently alike and that the oxygen equilibrium of each, taken alone, is characterized by a constant l . Let us assume, however, that in the native hemoglobin molecule the centers are subject to interactions whose free energies are additive but which are otherwise unrestricted. Let the interaction between centers 1 and 2 be α_{12} , that between 1 and 3 α_{13} , etc.⁵ In all there are six such constants, corresponding to the six ways of selecting two things from four, regardless of order. Then we can at once write the following expressions for the three L 's.

$$(4) \quad L_1 = l$$

$$L_2 = l^2 (\alpha_{12}\alpha_{13}\alpha_{23} + \alpha_{12}\alpha_{24}\alpha_{34} + \alpha_{13}\alpha_{14}\alpha_{24} + \alpha_{13}\alpha_{14}\alpha_{34})$$

$$L_4 = l^4 \alpha_{12}\alpha_{13}\alpha_{14}\alpha_{23}\alpha_{24}\alpha_{34}$$

We now introduce four new variables

$$(5) \quad A_1 = \alpha_{12}\alpha_{23}\alpha_{34}, \quad A_2 = \alpha_{13}\alpha_{14}\alpha_{24}, \text{ etc.}$$

⁵ It follows, of course, thermodynamically that $\alpha_{12} = \alpha_{21}$.

one for each of the four terms in the parentheses of the expression for L_3 . In terms of these variables

$$L_3 = P (A_1 + A_2 + A_3 + A_4)$$

and

$$L_4 = P^4 \sqrt{A_1 A_2 A_3 A_4}$$

The condition for symmetry then becomes

$$4\sqrt{A_1 A_2 A_3 A_4} = A_1 + A_2 + A_3 + A_4$$

or

$$(6) \quad 256 A_1 A_2 A_3 A_4 - (A_1 + A_2 + A_3 + A_4)^4 \equiv F = 0$$

At first glance it would seem to be impossible to determine the four A 's from this one equation, but this turns out not to be the case, owing to the physical requirement that they must all be real positive quantities.

It will be seen that the F of Equation 6 is a homogenous function (of the fourth degree) of the four variables. At best, therefore, it can only be possible to determine three of them in terms of the remaining one. Regarded as a function of any one of the four A 's, each of which occurs in it in exactly the same way, F is continuous. So are its first derivatives, *e.g.*,

$$\frac{\partial F}{\partial A_1} = 256 A_2 A_3 A_4 - 4(A_1 + A_2 + A_3 + A_4)^3$$

as well as its second derivatives, *e.g.*

$$\frac{\partial^2 F}{\partial A_1^2} = -12(A_1 + A_2 + A_3 + A_4)^2$$

For all positive values of the other three variables F is negative for $A_1 = 0$ and it certainly becomes negative as $A_1 \rightarrow \infty$; also $\partial F / \partial A_1$. At the same time $\partial^2 F / \partial A_1^2$ is uniformly negative for $A_1 \geq 0$. It follows from this that F , regarded as a function of A_1 , can have at most only one maximum for $A_1 > 0$, depending on the value of the other variables. Exactly the same considerations apply to each of the other A 's. At most, if we expand Equation 6, we see that there can be only two changes of sign in the coefficients of any A , depending on the values of the other variables, and therefore only two real positive roots. Whether or not such roots exist will depend on whether or not the maximum value of F (if there is one) with respect to the A in question is positive. Now let us consider the value of F which is a maximum with respect to all four A 's. This is determined by the four equations

$$(7) \quad \frac{\partial F}{\partial A_1} = 256A_2A_3A_4 - 4(A_1 + A_2 + A_3 + A_4)^3 = 0$$

$$\frac{\partial F}{\partial A_4} = 256A_1A_2A_3 - 4(A_1 + A_2 + A_3 + A_4)^3 = 0$$

It follows from these equations that this absolute maximum occurs when

$$(8) \quad A_1A_2A_3 = A_2A_3A_4 = A_1A_3A_4 = A_1A_2A_4$$

This demands that

$$(9) \quad A_1 = A_2 = A_3 = A_4$$

For this condition, however, it may easily be verified that F vanishes. For any other set of real positive values of A , F is negative. We may think of F as plotted in five-dimensional space as a function of the four A 's. Then (restricting ourselves to positive values of A) we see that the F surface lies everywhere below the four-dimensional plane $F = 0$,

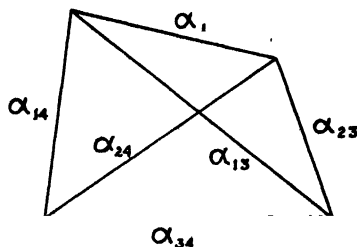


FIG. 6. For significance of this figure, see the argument regarding symmetry in the text.

except along the line of intersection of this plane and the two-dimensional plane $A_1 = A_2 = A_3 = A_4$, where its value is a maximum. All this means that a solution of the equation of symmetry with real positive values of A occurs if, and only if, the four A 's are all equal to one another. If we now substitute for A its values in terms of the six α 's, we obtain by straightforward algebra the result

$$(10) \quad \alpha_{12} = \alpha_{34}, \alpha_{23} = \alpha_{14}, \alpha_{13} = \alpha_{24}$$

This may be visualized by representing the four oxygen-combining centers by the vertices of a four-sided figure (see Fig. 6). Then if distances are taken to represent interactions, we have a picture of the symmetry of any arrangement which corresponds to the symmetry of the equilibrium curve. The figure of course need not be plane.

As we pointed out earlier, if the inherent properties of the four oxygen-combining centers were not identical, there would have to be a most im-

probable and, in fact, almost inconceivable set of compensating effects regarding the interaction constants to give rise to symmetry. We believe, therefore, that the present study, based on the new method, points to the presence of a very fundamental type of symmetry in the arrangement of the hemes in hemoglobin. More than this, the virtual identity in the value of n for such diverse pigments as the hemoglobin of horse, man, sheep, and bullfrog further suggests some common pattern of symmetry in the arrangement of the hemes in all the vertebrate hemoglobins.

SUMMARY

A simple spectrophotometric method is described, which was developed primarily for the study of the oxygen equilibrium of hemoglobin, but which is also applicable to equilibria with other gases. This is believed to be more accurate than other methods and can be used on small amounts of solution (1 cc. or less) of the highest attainable concentrations. This method has been employed to establish (1) the invariance of shape with pH of the equilibrium curves in which the fractional saturation of hemoglobin is plotted against the logarithm of the partial pressure of oxygen and (2) the complete symmetry of such curves obtained on concentrated solutions of freshly prepared protein. These results have been obtained on all types of hemoglobin studied. The first result (invariance of shape of the curves) indicates that the four oxygen-combining centers are all inherently identical, according to an argument developed elsewhere, and may be taken to mean that the heme-globin linkage is the same for each heme. The second result indicates the complete equivalence of the four centers regarding their mutual interactions and presumably, therefore, their special relations to one another, according to an argument developed in this paper. The identity of shape (as indicated by the value of n) of curves obtained on a variety of different vertebrate hemoglobins suggests a common arrangement of the four hemes in all cases.

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ISOLATION OF 17-HYDROXYCORTICOSTERONE FROM BLOOD OBTAINED FROM ADRENAL VEINS OF DOGS*

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In a previous publication (1) the isolation of a steroid hormone from blood in the adrenal veins of dogs after the administration of adrenocorticotrophic hormone (ACTH) was described. This hormone appeared to be Compound F of Dr. E. C. Kendall, 17-hydroxycorticosterone. Since then the isolation has been repeated with larger amounts of blood and with some difference in methods, and the identity of the compound has been established.

EXPERIMENTAL¹

The left adrenal veins of four heparinized dogs anesthetized with nembutal were cannulated. Each dog was then injected with 5 mg. of Armour's ACTH (Lot 57-A)² and the blood was collected continuously for 2 to 4 hours. The volumes of blood collected from these animals were 225, 200, 470, and 825 ml. Each sample was diluted with an equal amount of water and extracted with five portions of ether, of equal volume to that of the laked blood. The extraction was carried out by agitation in large centrifuge bottles, the mixture was centrifuged, and the ether layer taken off. The five ether extracts from each sample of blood were combined and evaporated to dryness. The dried ether residues weighed 388, 440, and 305 mg., and 1.276 gm., respectively.

The first residue was dissolved in 28 ml. of hexane and shaken with 40 ml. of 70 per cent ethanol. The ethanol solution was extracted with two 28 ml. portions of hexane. Then the hexane solutions were combined and

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‡ Postdoctoral Fellow, National Cancer Institute.

¹ All melting points were determined on the Kofler micro hot stage and are corrected.

² We are indebted to Dr. E. E. Hays and Dr. C. W. Mote of Armour and Company for kindly supplying this material.

extracted again with 40 ml. of 70 per cent ethanol. This ethanol solution was then washed with two 28 ml. portions of hexane. The hexane solutions were combined and kept for further investigations. The two 70 per cent ethanol solutions were also combined. The second residue was worked up in the same manner, while, for the third, 56 ml. portions of hexane and 80 ml. portions of 70 per cent ethanol were used. The 70 per cent ethanol solutions from these three fractions (approximately 320 ml.) were combined and concentrated *in vacuo* under nitrogen to 136 ml. The aqueous suspension then was extracted with three 136 ml. portions of ethylene dichloride. The separation was very slow and gave some emulsion which could be broken by centrifugation and filtration. The clear ethylene dichloride solutions were combined and evaporated *in vacuo*. After drying, the residue weighed 380 mg.

The ether extract from the blood of Dog 4 (1.276 gm.) was distributed between hexane and 70 per cent ethanol in the manner described above. In this case 84 ml. portions of hexane and 120 ml. portions of ethanol were used. The ethanol solutions (approximately 240 ml.) were combined, concentrated to 85 ml., and extracted with three 85 ml. portions of ethylene dichloride. The residue from the combined ethylene dichloride solutions weighed 243 mg.

Both ethylene dichloride extracts were combined (623 mg.), dissolved in 30 ml. of hexane, and extracted twelve times with 4 ml. portions of 30 per cent methanol. The 30 per cent methanol solutions were passed through two more funnels with 30 ml. of hexane each. The hexane solutions were completely clear, while the 30 per cent methanol solutions formed thick emulsions. The hexane solutions were combined and gave 474.3 mg. of residue on evaporation.

The 30 per cent methanol solutions were also combined and evaporated *in vacuo*. The residue weighed 137.3 mg. This material was dissolved in 2 ml. of methanol and 0.1 ml. of glacial acetic acid, and 175 mg. of Girard's Reagent T were added. The mixture was kept at room temperature overnight, poured in a mixture of ice water and 0.84 ml. of 2 N NaOH, and extracted three times with cold ethyl acetate. The "non-ketones" weighed 92 mg. To the aqueous solution, 10 per cent of the volume of concentrated HCl was added and the mixture was allowed to stand at room temperature for 24 hours. Then it was extracted three times with ethyl acetate. From these solutions 18.3 mg. of so called A-ketones were isolated. A second separation, carried out by refluxing the "non-ketone" fraction with Girard's reagent for 1 hour, gave 76.2 mg. of non-ketones and 9.0 mg. of B-ketones. The A-ketones showed strong absorption at 240 $m\mu$, while the B-ketones showed no absorption maxima. The A-ketones (18.3 mg.) were acetylated with 1 ml. of pyridine and 0.5 ml. of acetic anhydride

overnight at room temperature. The crude acetate weighed 15.5 mg. and was partly crystalline. After washing with two 5 ml. portions of hexane-benzene 1:1, the crystals weighed 5.6 mg., m.p. 190–209°. The two fractions, crystals and mother liquor, were chromatographed separately with 200 and 465 mg. of activated neutral aluminum oxide (Harshaw), respectively. The eluates obtained in the chromatography of these fractions with benzene-ether mixtures from 9:1 to 1:1 gave crystals which were

TABLE I

Melting Points and Ultraviolet Absorption Maxima of Crystalline Fractions Obtained from Chromatogram 1 (Crystalline Material)

Fraction No.	Solvent	M.p.	Ultraviolet maximum
		°C.	m μ
6	Benzene-ether 9:1	112– 140.5	240 (Weak)
7	“ 4:1	184– 212	240 (Strong)
8	“ 7:3	213.5–221.5	
9	“ 3:2	208– 221	
10	“ 1:1		240 (Strong)

TABLE II

Melting Points and Ultraviolet Absorption Maxima of Crystalline Fractions Obtained from Chromatogram 2 (Mother Liquor)

Fraction No.	Solvent	M.p.	Ultraviolet maximum
		°C.	m μ
6	Benzene-ether 9:1	199– 218.5	238 (Weak)
7	“ 4:1	127– 143	240 (Strong)
8	“ 7:3	117– 214	240 “
9	“ 3:2	206.5–220.5	240 “
10	“ 1:1	196– 218	
11	“ 2:3	Traces	240 (Strong)

washed with ether, ether-pentane 1:1, and pentane. The washings were saved.

The melting points and ultraviolet absorption maxima of the crystalline fractions obtained from Chromatograms 1 (crystalline material) and 2 (mother liquor) are presented in Tables I and II respectively.

Fraction 8 from Chromatogram 1 mixed with the acetate^a of Compound F (m.p. 212–220.5°) melted at 214–221.5°, with the acetate of Compound E (m.p. 215.5–220°) at 197.5–209°. The fluorescence reaction with con-

^a We are grateful to Dr. H. L. Mason for furnishing an authentic sample of the acetate of Compound F and for carrying out the fluorescence reaction with the acetate isolated from blood in the adrenal vein.

concentrated sulfuric acid, characteristic for Compound F and its acetate, was strongly positive. Synthetic Compound E does not give this reaction, while Compound E isolated from adrenal glands shows green fluorescence, probably owing to the presence of some Compound F which cannot be removed by recrystallization (2).

Fractions 9 and 10, and the pentane-ether washings of Fraction 8 of Chromatogram 1, were combined with Fractions 9 through 11 of Chromatogram 2. The combined material weighed 13.6 mg. It was rechromatographed with 150 mg. of Al_2O_3 . By development with benzene-ether 7:3 and 3:2, fractions were obtained which gave crystals, m.p. 213.5–219.5° and 207–217.5° respectively. They were combined with the crystals of Fraction 8 of Chromatogram 1 (3.2 mg. altogether) and saponified with 2.5 mg. of KHCO_3 in 0.46 ml. of methanol and 0.045 ml. of water (20 hours at room temperature). The saponified material weighed 2.8 mg. Samples of this material were analyzed by the paper chromatographic methods developed for the identification of adrenocortical steroids in microgram quantities (3, 4). Chromatograms in toluene-propylene glycol, developed for 3 and 7 days, gave a single spot of identical mobility to that of Compound F. An aliquot was acetylated and chromatographed in benzene-formamide for 18 hours. A single spot was found of mobility identical to that of the acetate of Compound F. Similar results were obtained when a sample was esterified with propionic anhydride and chromatographed in benzene-formamide for 6 hours. A spot of mobility identical to that of an authentic specimen of the propionate of Compound F was found. In all three cases the following reagents were employed to detect the spots: (a) alkaline ammoniacal silver nitrate reagent (α -ketol), (b) a solution of 2,4-dinitrophenylhydrazine in dilute acid (α,β -unsaturated ketone), and (c) concentrated sulfuric acid (11-hydroxy group). In the last case a thin layer of concentrated sulfuric acid was applied to a glass plate and the filter paper strip was laid on it. A green fluorescent spot indicated the position of Compound F or its esters.

Another sample of the material isolated from blood was acetylated and treated with chromic acid in glacial acetic acid at room temperature. After dilution with water the oxidized ester was extracted with ethyl acetate and chromatographed on paper in benzene-formamide for 16 hours. It gave a spot of mobility identical to that of an authentic specimen of the acetate of Compound E.

When a small amount of the natural material was oxidized with chromic acid in glacial acetic acid, and the reaction product chromatographed in toluene-propylene glycol for 4 hours, a spot moving at the same rate as that of an authentic specimen of adrenosterone was obtained. 2,4-Dinitrophenylhydrazine in dilute acid and an alkaline solution of *m*-dinitrobenzene in alcohol (Zimmermann reaction) were used for the detection of the spot.

Small amounts of the natural material and of Compound F were treated in parallel experiments with periodic acid solution. Samples of the two reaction mixtures were chromatographed in *n*-butanol-water and *n*-butanol-10 per cent NH_3 . The derivative from the natural material behaved in exactly the same manner as did that obtained from Compound F. In the first chromatogram it moved with the solvent front and in the second at an R_f of about 0.30. The retardation in movement produced by the use of 10 per cent NH_3 denotes the presence of an acidic group in the degradation product. The material gave an orange-red color with 2,4-dinitrophenylhydrazine reagent (α,β -unsaturated ketone) and a green fluorescent spot with concentrated sulfuric acid (11-hydroxy group). The degradation product was undoubtedly Δ^4 -3-keto-11 β ,17 α -dihydroxyetiocholenic acid.

Another sample was purified by chromatography with toluene-propylene glycol for 3 days and eluted from the paper with methanol which was subsequently evaporated. Concentrated H_2SO_4 was then added to give an approximate concentration of 40 γ of steroid per ml. of acid. The solution was allowed to stand at room temperature for 1 hour and then analyzed in a Beckman spectrophotometer. The absorption spectrum showed maxima at 238, 280, 395, and 475 μ . The same maxima were found for the chromogen obtained by similar treatment of Compound F. On the other hand, the five other active adrenocortical hormones and six related compounds which have been studied so far (5) gave chromogens whose absorption curves were different.

Δ^4 -3-Keto-11 β ,17 α -dihydroxyetiocholenic Acid Methyl Ester—Since only 0.8 mg. of the natural material was used for paper chromatographic analysis, approximately 2 mg. were still available and were used to prepare the etio acid methyl ester for melting point determination and infra-red analysis. They were dissolved in 0.28 ml. of dioxane, and a solution of 3.1 mg. of $\text{HIO}_4 \cdot 2\text{H}_2\text{O}$ in 0.1 ml. of water was added. After standing at room temperature for 20 hours, the mixture was diluted with ether and washed twice with 2 *N* sodium carbonate solution and once with water. The neutral material from the ether solution weighed 0.9 mg. The alkaline solutions were acidified and extracted four times with CHCl_3 . The CHCl_3 solutions were washed with a little water, dried, and evaporated. The residue weighed 1.0 mg. and was esterified with diazomethane. 1.1 mg. of methyl ester were obtained. This material was chromatographed on 150 mg. of Al_2O_3 (Merck). Most of the substance was found in the fractions eluted with benzene-ether 3:2, 1:1, and 2:3. From ether, needles were obtained which melted between 200–206°. Δ^4 -3-Keto-11 β ,17 α -dihydroxyetiocholenic acid methyl ester melts at 207–208° (6). Since no authentic specimen of this ester was available, no direct comparison could be made. The infra-red spectrum in carbon disulfide taken on a Beckman

spectrophotometer model IR2 showed peaks at 5.77μ (etio acid methyl ester), 5.95μ (Δ^4 -3-keto group), and 2.70μ (hydroxyl group) (7). In the ultraviolet the substance showed strong absorption at $240 m\mu$.

Δ^4 -3,11-Diketo-17 α -hydroxyetiocholenic Acid Methyl Ester—For comparison Δ^4 -3,11-diketo-17 α -hydroxyetiocholenic acid methyl ester, which is not described in the literature, was prepared by oxidation of Compound E with periodic acid. The acid thus obtained was methylated with diazomethane and the crude methyl ester was chromatographed on Al_2O_3 (Merck). The whole material was eluted with benzene-ether 9:1, 4:1, and 7:3. After recrystallization from acetone-hexane, prisms were obtained which melted at 229 – 231° .

Analysis— $C_{21}H_{28}O_5$.	Calculated.	C 69.98, H 7.83
	Found.	" 69.94, " 8.01

Since this substance was too insoluble in CS_2 , no infra-red spectrum could be taken in this solvent. In chloroform, three peaks in the ketone region at 5.84 , 9.95 , and 6.01μ were found, corresponding to the carbomethoxy group, the 11-keto group, and the Δ^4 -3-keto group. It is known (8) that in $CHCl_3$ solution the maxima are shifted to longer wave-lengths.

SUMMARY

Free 17-hydroxycorticosterone (Compound F of Kendall) was isolated from blood obtained from the adrenal veins of dogs after administration of adrenocorticotrophic hormone.

During the identification of the product of periodic acid oxidation, Δ^4 -3,11-diketo-17 α -hydroxyetiocholenic acid methyl ester, not previously described, was prepared.

Addendum—Since this paper was submitted for publication, Fractions 6 and 7 of Chromatogram 1 and Fractions 7 and 8 of Chromatogram 2 were combined and analyzed by paper chromatography. This combined material was found to be a mixture containing approximately 1 part of the acetate of Compound F and 3 parts of the acetate of corticosterone. The acetate of corticosterone was identified as follows: (1) A mixed chromatogram of this material with an authentic sample of the steroid acetate gave a single spot after treatment with alkaline triphenyltetrasolium chloride, 2,4-dinitrophenylhydrazine, or concentrated sulfuric acid. (2) The chromogen obtained when the isolated compound was treated with concentrated sulfuric acid showed maxima at 285 , 330 , 373 , and $455 m\mu$, indistinguishable from those given by the chromogen of pure acetate of corticosterone (5). (3) After mild oxidation with chromic acid in glacial acetic acid, the isolated compound gave a product whose chromatographic behavior was identical with that of the acetate of 11-dehydrocorticosterone. The oxidation product gave a sulfuric acid chromogen with maxima

⁴ The analysis was carried out by Dr. G. Weiler and Dr. F. B. Strauss, Oxford, England.

at 280, 355, and 415 $m\mu$, characteristic of 11-dehydrocorticosterone (5). The presence of the acetate of corticosterone is also indicated by the melting points of Fraction 6 of Chromatogram 1 and Fraction 7 of Chromatogram 2. The pure acetate of corticosterone has a double melting point at 145-146° and 152-153° (2).

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THE CONVERSION OF TRYPTOPHAN TO KYNURENINE IN LIVER*

I. THE COUPLED TRYPTOPHAN PEROXIDASE-OXIDASE SYSTEM FORMING FORMYLKYNURENINE

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The enzyme reactions bringing about oxidation in the indole ring of tryptophan and kynurenine formation furnish a convenient example of those poorly understood systems producing aromatic ring oxidations. The metabolism of tryptophan via kynurenine has been studied in different instances by nutritional, isotopic, genetic, and adaptive experiments; thus this metabolic pathway has been well documented on animals (1, 2), bacteria (3-5), *Neurospora* (6), and insects (7, 8). The enzymes responsible for these reactions have received little attention. There are only the reports of Kotake and his school ascribing the reaction to a "tryptophan-pyrrolase" found in liver. The demonstration of this, by day long incubation under paraffin (9), yielded no definite reaction in our hands.

We have been able to demonstrate kynurenine formation from tryptophan in liver homogenates under aerobic conditions (10). In this paper the over-all reaction to kynurenine is described, and the first two steps are identified as a physiological, coupled oxidation system consisting of a tryptophan peroxidase and an oxidase which forms peroxide. Tryptophan oxidation by this system yields formylkynurenine. The identification of formylkynurenine and of the enzyme formylase which hydrolyzes it to formic acid and kynurenine is described in Paper II of this series (11).

Methods

Preparation of Enzymes

Livers removed immediately after death are homogenized in twice their weight of ice-cold 0.9 per cent KCl, according to the procedure of Green

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(12). The supernatant solution obtained after centrifugation for 15 minutes at $11,000 \times g$ is substantially free of mitochondrial elements and is referred to as the crude enzyme preparation.

The best relative separation of the tryptophan peroxidase-oxidase system from the formylase is achieved with animals which have been given 1 to 2 gm. of DL-tryptophan per kilo about 6 hours before death. This treatment, to be described elsewhere, produces a 10-fold increase in the activity of the liver oxidizing system and none in the formylase, which is already present in excess. The crude enzyme is then diluted with 4 volumes of water and the precipitate is centrifuged and discarded. One-third of the original volume of 0.5 M KH_2PO_4 is added, followed by dropwise addition of 1 per cent acetic acid in 0.5 M KH_2PO_4 to bring the pH to 5.4. After rapid centrifugation, the supernatant solution is neutralized with 1 N NaOH, and the precipitate is washed twice with the original volume of 0.1 M acetate buffer, pH 5.4. The precipitate is then dissolved and neutralized with 0.2 M Na_2HPO_4 and diluted to one-half the original volume. Large losses in activity are encountered if the temperature is not maintained below 5° .

The D-amino acid oxidase preparation used was a centrifuged 10 per cent extract of pig kidney acetone powder made up fresh every 3 days. In 30 minutes, 0.2 ml. of this preparation catalyzes the uptake of $2 \mu\text{M}$ of oxygen with 0.05 M DL-alanine as substrate. Armour's "powdered catalase" was used throughout, 0.1 ml. of a 1 per cent solution having approximately the catalase activity of 1 ml. of the supernatant solution from rabbit liver. A thick suspension of the mitochondrial fraction of rat liver, washed several times with water to inactivate many of the other systems present, was used as the uricase preparation. A preparation of formylase free of catalase was used, 1 ml. of which hydrolyzed $300 \mu\text{M}$ of formylanthranilic acid per hour. All these enzyme preparations were themselves completely inactive in forming kynurenine from tryptophan.

Assays

Aerobic incubation of the enzyme reaction mixture was routinely carried out in 60 ml. bottles in a rack attached to the shaking mechanism of the Warburg bath or in standard manometer vessels, and the activity assayed by determination of the kynurenine formed. Blanks without substrate, included in each experiment, were low even after treatment of the animal with tryptophan, and the values of these were subtracted from those containing 0.0025 M L-tryptophan. 2 ml. aliquots were deproteinized by addition of 2 ml. of 5 per cent zinc acetate and 2 ml. of 0.18 N NaOH. The kynurenine in the filtrate, determined by its absorption at $360 \text{ m}\mu$ in

a 1 cm. cell in a Beckman spectrophotometer, is calculated on the basis of a density reading of 0.440 for 0.1 μM per ml. This value is quite constant over the range pH 4 to 8. Assay of kynurenine by diazotization at 0° was made by the method of Bratton and Marshall (13) on a 5 per cent trichloroacetic acid filtrate to avoid the color development with tryptophan occurring in stronger acid (14), and the color was read in a Coleman junior spectrophotometer at 560 $\text{m}\mu$ after 60 minutes. The Schiff base of kynurenine is developed from 2 ml. of the neutral filtrate, 1 ml. of 1 M phosphate buffer, pH 1.2, and 0.5 ml. of 2 per cent *p*-demethylaminobenzaldehyde in ethyl alcohol. The yellow color formed is read at 460 $\text{m}\mu$ in the Beckman spectrophotometer at 24° and provides a more rapid but less sensitive chemical assay than the diazotization procedure. A reading of 0.130 density is obtained with 0.1 μM of kynurenine. This reaction has a sharp pH optimum and is very sensitive to temperature; the color decreases with a rise of temperature.

The kynurenine used as a standard was prepared by the method of Butenandt *et al.* (7).

Tryptophan and kynurenine cannot be clearly separated by paper chromatography with butanol or phenol even in the presence of NH_3 . Development with *n*-butanol saturated with 0.1 N HCl , however, gives R_F values of 0.29 for kynurenine and 0.40 for tryptophan under our conditions (ascending solvent). Amino acid spots are developed by application of ninhydrin in pyridine.

Tryptophan was determined in duplicate with the Hopkins-Cole (15) and Lugg (16) methods. Kynurenine does not interfere. Higher readings were obtained in both methods from tryptophan plus enzyme than from tryptophan alone, although the enzyme blanks were insignificant. Standard tryptophan values were therefore determined after addition to the enzyme. Formic acid was determined by the method of Grant (17).

Demonstration of Kynurenine Formation from Tryptophan

When L-tryptophan is incubated aerobically with the supernatant of a fresh liver homogenate, tryptophan disappears, oxygen is taken up, and a diazotizable aromatic amine is formed (Table I). No activity was found in other tissues. The magnitude of the reaction is normally small: liver preparations of all species studied, rabbit, rat, guinea pig, dog, pig, horse, and pigeon, showed activity only of the order of 1.5 μM of tryptophan converted per gm. of wet weight of liver per hour. The activity of livers of animals given tryptophan before sacrificing, as described under "Preparation of enzymes," increases as much as 10-fold without any qualitative change in the reaction.

The measurements of the reaction in crude enzyme preparations given

in Table I show that for each mole of tryptophan disappearing 1 mole of diazotizable amine appears, if kynurenine itself is used as the standard for the amine determination. Approximately 1 mole of oxygen is consumed in this reaction, although the values shown are uncertain owing to the high blank of the enzyme preparation (see confirmatory oxygen uptake values of Table V). No CO_2 is produced, but 1 mole of formic acid appears with each mole of kynurenine; thus the following balance is obtained.



The amine formed in the reaction has been identified as kynurenine from its properties and by paper chromatography without actual isolation.

TABLE I

Stoichiometry of Oxidation of L Tryptophan by Soluble Liver Protein Fraction

In each vessel 1 to 2 ml. of crude enzyme, 0.2 mg. of methylene blue, 1 μM of adenosinetriphosphate, and 4 μM of MgSO_4 . Final volume was 4.0 ml., buffered at pH 7.5 with 0.025 M phosphate. 5 μM of L-tryptophan were included in the experiments in which tryptophan was determined; 10 μM were added in the others. All the determinations are corrected for blanks run without tryptophan. The reactions were carried out in vessels shaken at 38° for 30 or 60 minutes. The gas phase was air.

Experiment No.	— Δ tryptophan	Δ kynurenine	— Δ oxygen	ΔCO_2	Δ formic acid
	μM	μM	μM	μM	μM
1	1.9	1.5	2.9		
2	0.6	0.6	1.6		
3		3.2	3.8	0.0	
4		3.5	4.9	0.0	
5		0.8	1.9		
6		3.8			4.1
7		1.4			1.5

Its ultraviolet absorption curve with a maximum at 360 $\text{m}\mu$ is identical in shape at different pH values with that of kynurenine (7) and of the proper magnitude for kynurenine calculated from the chemical assay by diazotization. The blue product formed after diazotizing and coupling is identical with that from kynurenine in its absorption maximum at 560 $\text{m}\mu$ and in its rate of color development. This color, which reaches a maximum in 90 minutes under our conditions, is identical with that developing in 24 hours from anthranilic acid. The product gives the Otani reaction (8) with *p*-dimethylaminobenzaldehyde and H_2O_2 , characteristic of kynurenine and forms a yellow Schiff's base with *p*-dimethylaminobenzaldehyde at pH 1.2. The product can be separated from unchanged tryptophan by paper chromatography and the integrity of the α -amino group

demonstrated with ninhydrin. It forms a spot at the R_f value characteristic of kynurenine which fluoresces under ultraviolet light.

Specificity of System

Only the L isomer of tryptophan gives rise to an aromatic amine in the system. In early experiments it was shown that twice the concentration of DL- as of L-tryptophan was required to give the half maximal and full activity in the system. With more concentrated enzyme preparations, it was later possible to show that substantially all the L-tryptophan but only half of the DL-tryptophan reacted (Table II). Even substitution in the α -amino group of tryptophan prevents action by the enzyme system, since no amine is formed from acetyl-DL-tryptophan. Various other indole compounds were tested and also failed to give rise to aromatic

TABLE II

Kynurenine Formation from L- and DL-Tryptophan with Excess Enzyme

2 ml. of enzyme, 0.2 ml. of D-amino acid oxidase, 200 μ M of DL-alanine, and 0.025 M phosphate buffer at pH 7.0; total volume 4.0 ml. 120 minutes at 38° in air.

DL-Tryptophan added	L-Tryptophan added	Kynurenine formed
μ M	μ M	μ M
2		0.82
	2	1.57
4		1.65
	4	3.24

amines. Tryptamine, indole-3-acetic acid, indole-3-propionic acid, indole-3-butyric acid, skatole, oxindole, 1-ethyloxindole, dioxindole, acetonyl-3-hydroxyoxindole, and isatin all were inactive.

This specificity of the over-all reaction is equaled by the specificity of the first (oxidizing) reaction, as shown by oxygen uptake measurements. Our most active crude preparations fail to consume any extra oxygen in the presence of D-tryptophan, 1-methyl-DL-tryptophan (kindly provided by Dr. H. R. Snyder) or indolepropionic acid.

Properties of Over-All Enzyme System

Under the optimal conditions of assay, the oxygen uptake and kynurenine formation are proportional to the amount of enzyme and to the duration of the experiment. Activity is obtained between pH 6.5 and 8.5 with the optimum at about 7.5, which shifts to pH 7.0 on addition of an external source of peroxide. L-Tryptophan concentrations of 0.0004 M give half maximal and 0.001 M or more give maximal activity during 30

minute experiments. In a typical experiment 0.2 ml. of enzyme formed 0.33 μM ; 0.5 ml., 0.86 μM ; and 1.0 ml., 1.94 μM of kynurenine.

The reaction requires oxygen, and neither various dyes nor ferricyanide will act as an oxidant in the reaction anaerobically. Yet the addition of methylene blue or other autoxidizable dyes to the aerobic system will double the activity of a fresh rabbit enzyme preparation and triple or quadruple the lowered activity of the same preparation after it is aged or dialyzed. Further restoration of the activity lost on even short dialysis is obtained by addition of crude coenzyme fractions, in which the active substances have been identified as adenosine-5-phosphate and Mg^{++} . The effect of these factors is exerted only in the presence of methylene blue.

The crude enzyme preparation kept at 0° retains most of its activity for 1 to 2 weeks and for longer periods when kept frozen at lower temperatures. The activity is greatly decreased by aerobic incubation for 15 minutes in the absence of tryptophan. The activity is also easily lost upon attempts to separate the several reactions by fractionation. While the factors methylene blue, adenylic acid, and Mg^{++} have little effect in reactivating purified fractions of the system, the provision of an adequate source of enzyme-generated H_2O_2 , as demonstrated later, will frequently allow considerable activity to be recovered in such fractionations. Assayed in this way, the active oxidizing fraction is found to be precipitated from the crude enzyme at pH 5.4 and can also be fractionated between 30 and 50 per cent ammonium sulfate saturation or with 20 per cent ethanol at -5°. It is inactivated in 5 minutes at 55°. The fractions obtainable in these ways have been quite unstable, and the losses of activity entailed in even the best separations have prevented any purification beyond a 10-fold increase in oxidizing action per mg. of protein.

Requirement for Hydrogen Peroxide

The salutary effect produced on this aerobic system by methylene blue, which, after reduction by blank reactions in the crude enzyme, would be reoxidized with formation of H_2O_2 , suggested that peroxide might be required for the reaction. The tryptophan-oxidizing capacity of the crude system can be diminished in three different ways which presumably decrease endogenous peroxide production: by omitting methylene blue, by aerobic incubation to decrease the substrates of methylene blue-reducing systems before adding tryptophan, and by omitting the adenylic acid and Mg^{++} apparently necessary to reactivate some methylene blue-reducing system after dialysis. When another source of peroxide is added to the system inhibited in these ways, the activity is increased (Table III). A source of enzymically generated peroxide, such as uric acid (a small amount of uricase is present in the crude enzyme), uricase plus uric acid, or D-

amino acid oxidase plus DL-alanine, will produce this restoration of activity, but H_2O_2 itself will not. Provision of an adequate source of generated hydrogen peroxide in the assay system completely eliminates the requirement for methylene blue, adenylic acid, and Mg^{++} , and prevents that part of the loss sustained upon purification which is due to the elimination of endogenous peroxide-donating systems.

TABLE III

Requirement of Tryptophan-Oxidizing System for Hydrogen Peroxide

The original activity was measured with crude enzyme supplemented and tested as in Table I. The following quantities were used when indicated: 2 mg. of uric acid, 0.5 ml. of uricase, 18 mg. of DL-alanine, and 0.2 ml. of D-amino acid oxidase. All the values are expressed as micromoles of kynurenine formed per ml. of enzyme per hour.

Original activity Kynure- nine μM	Condition	Activity		Activity after adding source of peroxide to inhibited enzyme	
		Kynure- nine μM	Kynure- nine μM	Addition	Kynure- nine μM
0.46	Methylene blue omitted	0.31	0.31	10 μM H_2O_2	0.31
1.01	"	0.39	0.39	Uric acid	0.57
1.53	"	0.56	0.56	" " + uricase	1.36
1.42	"	0.98	0.98	" " + "	1.31
				" " + "	1.54
				+ methylene blue	
0.55	"	0.21	0.21	DL-Alanine + D-amino acid oxidase	0.54
0.60	"	0.24	0.24	" + "	0.54
0.46	Preincubated at 37°	0.21	0.21	10 μM H_2O_2	0.21
1.68	" 37°	0.51	0.51	Uric acid + uricase	0.95
	" 37°	0.33	0.33	DL-alanine + D-amino acid oxidase	0.67
1.20*	ATP† and Mg^{++} omitted	0.63	0.63	Uric acid + uricase	1.39
				" " + " + ATP + Mg^{++}	1.40

* Enzyme dialyzed overnight *versus* distilled H_2O .

† Adenosinetriphosphate.

The amounts of peroxide required by the crude system are small and comparable in amount to the tryptophan oxidized, although large amounts of catalase are present in the crude system. The oxidizing system can be separated from the catalase and separated at the same time from formylase, by precipitation at pH 5.4 (see the method described for the separation of the oxidase system from formylase). When the separation of formylase from such a precipitated fraction is sufficiently complete, tryptophan oxi-

dation gives rise to an accumulation of formylkynurenine (11). The oxidation of tryptophan to formylkynurenine by such a precipitated fraction occurs at a rate not affected by addition of formylase to carry the reaction on to kynurenine (Table V). Purified formylase, in excess, can therefore be added to the precipitated fraction in order to follow the oxidation reaction in the absence of catalase (Table IV).

TABLE IV

Inhibition of Tryptophan Oxidation in Precipitate at pH 5.4 by Either Catalase or H_2O_2

1 ml. of three different enzyme preparations at pH 5.4, 0.05 ml. of formylase, 0.5 ml. of 0.2 M phosphate buffer at pH 7.0; total volume 4.5 ml., 30 minutes incubation. 10 μ M of L-tryptophan in experimental vessels. Additions of D-amino acid oxidase were accompanied by 0.2 ml. of 1 M DL-alanine. D-Amino acid oxidase, 1 per cent catalase, and 0.01 M H_2O_2 (0.1 ml. at 5 minute intervals) added as indicated in the amounts in parentheses.

Additions to ppt.	Kynurenine formed by ppt. alone		
	Experiment 1	Experiment 2	Experiment 3
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
pH 5.4 alone	100	100	100
Catalase	46 (0.05 ml.)	31 (0.2 ml.)	
“ + D-amino acid oxidase	118 (0.05 “)		
“ “	122 (0.2 “)	92 (0.2 “)	135 (0.1 ml. catalase + 0.2 ml. D-amino oxidase)
“ “	119 (0.6 “)		120 (0.1 ml. catalase + 0.5 ml. D-amino oxidase)
Catalase + H_2O_2		37 (0.2 ml. + 6 μ M)	
H_2O_2		21 (6 μ M)	
D-Amino acid oxidase	59 (0.2 ml.)	61 (0.2 ml.)	59 (0.2 ml.)
“ “ “	60 (0.05 “)		

In such a reconstituted system, the requirement for peroxide by the tryptophan peroxidase can now be shown in another way. Addition of catalase diminishes the reaction, and this catalase inhibition is specifically reversed by addition of an external source of peroxide, but not by peroxide itself. The oxidative system is also sensitive to any slight excess of peroxide and is consequently inhibited by peroxide added in the absence of catalase. Catalase protects the system against this inhibition by any excess of generated peroxide, but not against the direct addition of peroxide. The addition of both catalase and a source of peroxide thus negates the inhibitory effects of either separately and provides the optimal condition for the reaction. Increasing the amounts of both peroxide and

catalase, together, by 12-fold does not further influence the reaction. The function of catalase in the system is limited to this protective action against excessive peroxide concentrations.

The sensitivity of the system to peroxide and to other more typical peroxidase inhibitors is also consistent with the existence of a tryptophan peroxidase which is able to use the small concentrations of peroxide existing in the presence of catalase. The reaction is completely inhibited by

TABLE V

Oxygen Consumption and Kynurenine Formation of Acid Precipitate

2.0 ml. of enzyme preparation at pH 5.4, 0.5 ml. of 0.2 M phosphate buffer, pH 7.0; total volume 4.0 ml., 60 minutes incubation. Oxygen consumption extrapolated to correct for the first 10 minute equilibration. Additions of 0.4 ml. of D-amino acid oxidase, 0.2 ml. of 1 M DL-alanine, and 0.2 ml. of 1 per cent catalase and a small amount of ethanol to all except the last experiment. 0.1 ml. of formylase added as indicated. 10 μ M of L-tryptophan added to experimental vessels. All values are expressed in micromoles.

Experiment No.		Oxygen uptake			Kynurenine formed	Ratio, O ₂ /kynurenine
		Enzyme blank	L-Tryptophan	Δ O ₂		
		μM	μM	μM	μM	
1	pH 5.4 enzyme.....	6.90	9.50	2.60	1.78 (3.89)*	0.67
2	" " " + formylase....	7.65	10.40	2.75	3.36	0.82
3	" " "	9.40	14.21	4.81	2.52 (5.73)*	0.84
4	" " " + formylase....	9.51	14.02	4.51	5.76	0.78
5	(No D-amino acid oxidase or catalase.) pH 5.4 enzyme + formylase.....	0.67	7.30	6.63	5.96	1.11

* Kynurenine determination after addition of formylase to the reaction mixture at the end of the experiment.

0.001 M cyanide, hydrogen sulfide, and arsenite and is strongly inhibited by the same concentrations of NaF, hydroxylamine, and sodium azide.

Demonstration of Coupled Tryptophan Peroxidase-Oxidase System

One of the two oxidation steps between tryptophan and formylkynurenine is catalyzed by a peroxidase, as revealed by the requirement for peroxide in the presence of catalase. Since the reaction can nevertheless occur in the catalase-free precipitated fraction without an added source of peroxide, the second oxidation step must form peroxide for the first. This can be demonstrated in a system free from catalase, if it has a sufficiently low blank oxygen uptake to preclude an adequate supply of H₂O₂ to the reaction, except from that arising from the oxidation of tryptophan itself. In Experiment 5 of Table V, the 0.7 μ M of blank oxygen consumption in

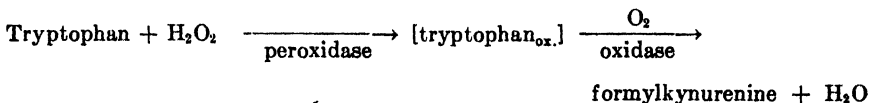
the system could supply at most only $1.4 \mu\text{M}$ of H_2O_2 to the tryptophan oxidation reaction. Yet, in the presence of tryptophan, an additional oxygen uptake totaling $6.6 \mu\text{M}$ occurred and $6.0 \mu\text{M}$ of kynurenine were formed. A total of $6.0 \mu\text{M}$ of H_2O_2 were therefore utilized in the peroxidase step, at least $4.6 \mu\text{M}$ of which could only have been produced by the second oxidation step in the reaction itself.

The high blank oxygen uptakes in Experiments 1 and 2 of Table V are due to the inclusion of ethanol along with D-amino acid oxidase and catalase in order to test the effect on the coupled oxidation of tryptophan of its prototype reaction, the coupled oxidation of ethanol by catalase (18). The comparable efficiency of H_2O_2 utilization in these two systems can be seen from the failure of catalase to use all the peroxide available for its reaction and so prevent the oxidation of tryptophan.

DISCUSSION

Although we were unable to repeat their observations, it must be assumed that the formation of kynurenine in liver observed by Kotake and Iwao (2) occurred through the system described here, since a second system able to cause this reaction has not been found. If the "tryptophan-pyrrolase" reaction is really due to this tryptophan peroxidase-oxidase system, it is different from that previously described. D-Tryptophan is not converted to D-kynurenine, nor is kynurenine further oxidized to anthranilic acid, as reported. The intermediate formation of a dicarboxylic acid by opening of the indole ring between the nitrogen and α -carbon is also ruled out by the identification of formylkynurenine (11) which must result from an α, β opening of the ring.

Since methylene blue is not reduced by tryptophan anaerobically in the presence of an active tryptophan-oxidizing system, as might be expected of an oxidase, the coupled oxidation system may be provisionally formulated with the primary step by using peroxide and the second step by using oxygen, as shown in the following scheme.



The efficient coupling through peroxide of the two oxidative steps of tryptophan oxidation in this system demonstrates a physiological role for this type of "cyclic oxidation" discovered by Keilin and Hartree (18). In their reaction, catalase catalyzes the oxidation of ethanol to acetaldehyde by means of peroxide generated in the further oxidation of the acetaldehyde catalyzed by xanthine oxidase. A somewhat closer analogy exists in the oxidation of dihydroxymaleic acid by peroxidases and oxygen, which,

like the tryptophan oxidation, can be inhibited by catalase (19). Enzymically generated H_2O_2 and not the preformed reagent functions in the catalase system only because of concentration limits, as is clear from the work of Chance (20), and presumably the same is true in the tryptophan system. The addition of catalase to the tryptophan oxidation system insures the maintenance of only these low peroxide concentrations under experimental conditions *in vitro* and is not necessarily to be regarded as a function of catalase under physiological conditions.

The function of the tryptophan peroxidase in the coupled system has not been dissociated from the oxidase reaction, but two separate enzymes should for the present be assumed to exist. The participation of a peroxidase in the system is clearly indicated by the catalase inhibition, reversible by peroxide, and by the sensitivity of the system to typical peroxidase inhibitors, regardless of the properties of a second enzyme. The association of the peroxidase reaction with the peroxide-producing reaction has, however, prevented the demonstration of a stoichiometric utilization of added H_2O_2 . Assuming that the peroxidase reaction occurs first, the specificity demonstrated for the first oxidation step can be attributed to the peroxidase. In addition to its absolute specificity for L-tryptophan among the indole derivatives, it will also not oxidize benzidine, pyrogallol, or guaiacum, even with generated peroxide. Its function nevertheless confirms the belief that peroxidases would be found to be concerned physiologically with aromatic ring oxidations. The specificity and sensitivity to peroxide inhibition explain why it has not previously been found by the more usual peroxidase tests and recalls the properties of cytochrome *c* peroxidase (21) and of catalase (peroxidase action).

No information is available as to the nature of the intermediate tryptophan oxidation product. The inactivity of the oxindole and dioxindole compounds tested still does not preclude as the intermediate either 2-hydroxytryptophan (22) or the 2,3-dihydroxydihydrotryptophan which might result from addition of H_2O_2 to the double bond of the indole ring. However, a *Pseudomonas*, able to oxidize both tryptophan and kynurenine, does not utilize 2-hydroxytryptophan.¹

Although lactoperoxidase, veridoperoxidase, and horseradish peroxidase will oxidize tryptophan and can do this by generated peroxide in the presence of catalase, as will be described later, the red-brown product formed is not kynurenine nor is it converted into kynurenine by an active crude liver enzyme system. Addition of these peroxidases to the liver system oxidizing tryptophan, in which the oxidation reaction is limiting, does not increase the kynurenine formation.

¹ Hayaishi, O., personal communication.

SUMMARY

1. A soluble enzyme system has been obtained from the livers of several species which converts L-tryptophan to kynurenine and formic acid. Only L-tryptophan will react in this system.

2. The system carrying out the first two oxidative steps to yield formyl-kynurenine can be separated from the hydrolyzing enzyme, formylase, and from catalase.

3. The oxidizing system consists of coupled peroxidase and oxidase reactions which have not been dissociated.

4. The tryptophan peroxidase differs from some other peroxidases in its specificity for this physiological reaction, in its requirement for slowly generated peroxide, and in the type of tryptophan oxidation produced. It can operate in the presence of catalase, which also protects it from inhibition by excessive peroxide concentrations.

We are indebted to Mr. Alexander Winterstein for technical assistance.

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THE CONVERSION OF TRYPTOPHAN TO KYNURENINE IN LIVER*

II. THE ENZYMATIC HYDROLYSIS OF FORMYLKYNURENINE

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In the oxidation of L-tryptophan to kynurenine by a liver enzyme preparation, formic acid also accumulates (1). This most probably arises from the hydrolysis of the previously unknown compound, formylkynurenine, suggested as an intermediate in this reaction by Heidelberger *et al.* (2). Formylkynurenine was also thought to be formed in the ozonolysis of tryptophan, from analogy with similar reactions, although it was not separated or identified (3).

Formylkynurenine has been prepared and its occurrence as an intermediate in the oxidation of tryptophan by liver is shown in the following way. The enzyme formylase, able to hydrolyze such a compound as formylkynurenine, was found in the liver system by the use of structural analogues of formylkynurenine. With the aid of an assay based on the use of this enzyme, formylkynurenine has been identified and separated from the product of tryptophan ozonolysis. Removal of the formylase from the liver system oxidizing tryptophan results in the accumulation of formylkynurenine which is identical with that isolated from the synthetic reaction.

Demonstration of Formylase

Formylanthranilic acid was chosen as an available substrate for determining the ability of liver preparations to hydrolyze aromatic formamides such as formylkynurenine. Hydrolysis of this compound to free anthranilic acid was demonstrated in crude rabbit liver enzymes by the Schiff's base method for determining the free amine (1). A half maximum rate is obtained with a rabbit liver preparation during hydrolysis for 15 minutes with 0.002 M and a maximum rate with 0.006 M formylanthra-

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nilic acid. The effect of pH is very small between pH 6 and 8, only a slight maximum occurring at pH 7.0. A more accurate assay was then developed, depending upon the difference in the ultraviolet absorption spectra of formylanthranilic acid and anthranilic acid at 330 $m\mu$ (Fig. 1), and when necessary neutral deproteinization ($Zn(C_2H_3O_2)_2-NaOH$) was used to avoid the slow spontaneous hydrolysis at acid pH. Purified enzyme preparations and even crude rat liver preparations are sufficiently clear to be

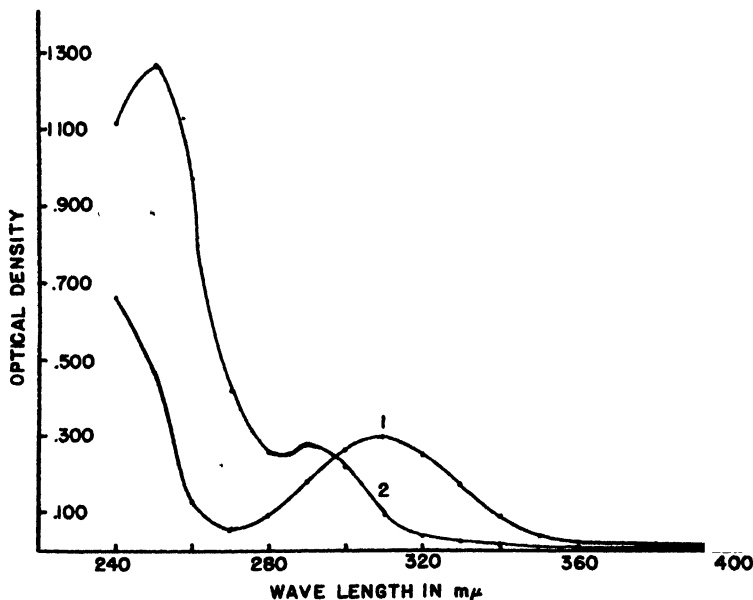


Fig. 1. Absorption spectra of anthranilic acid and formylanthranilic acid. Curve 1, anthranilic acid; Curve 2, formylanthranilic acid. Concentration of each 10^{-4} M, pH 7.5; depth of cell 1 cm.

assayed by this method directly in the spectrophotometer without preliminary incubation and deproteinization.

The assay is carried out in 1 cm. cuvettes containing 0.5 ml. of 0.2 M phosphate, pH 7.5, 0.3 ml. of 0.1 M formylanthranilic acid (prepared by dissolving with cautious neutralization), enzyme, and water in a total volume of 3 ml. Readings are taken at 30 second intervals after addition of the enzyme with stirring and against a blank without formylanthranilic acid, since there is no spontaneous hydrolysis of the substrate under these conditions. Amounts of enzyme are selected to give linear rates of hydrolysis for at least 1 minute. The rate subsequently diminishes, owing to the accumulation of anthranilic acid which slightly inhibits the reaction. For this reason the initial rates measured at approximately 25° by this

method are similar to the rates obtained during a longer period at 37°, followed by deproteinization before reading, as with cloudy and less active enzyme preparations. In this latter procedure the same reaction mixture is employed, and the density difference between filtrates of 0 and 20 minute aliquots is determined. Similar methods were used to follow the hydrolysis of most other compounds by this enzyme. The wave-lengths

TABLE I
Relative Rates of Hydrolysis by Formylase

Compound	Preparation*	Analytical method*	Wave-length	Δ per 0.1 μ m per cc.	Relative rate
Formylkynurenine	Tryptophan ozonolysis	Light absorption	360	0.440	100
Formylanthranilic acid	(5)	Schiff base	460		
		Light absorption	330	0.150	16
Formylorphanilic "	Sodium salt (cf. (5))	Schiff base	460		
		Light absorption	305	0.260	10
Formylnitroaniline	(6)	" "	490	0.040	10
Formyl- <i>m</i> -aminobenzoic acid	(7)	" "	320	0.070	3.7
Formanilide	(8)	Schiff base	460		
		Light absorption	295	0.047	2.6
<i>o</i> -Formaminophenol	(9)	Schiff base	460		2.6
Formyl- <i>o</i> -toluidine	(8)	Light absorption	295	0.054	1.8
Acetylanthranilic acid	Commercial	" "	330	0.150	1
Acetanilide	"	" "	290	0.052	0.4
Formyl- <i>p</i> -aminobenzoic acid	(10)	" "	305	0.100	0.4
Acetyl- <i>p</i> -aminobenzoic acid	Commercial	" "	305	0.100	0.3
Acetyl- <i>o</i> -aminophenol	"	Schiff base	460		0.2
Formylalanine	Gift from Dr. J. Greenstein	Ninhydrin (11)	570		0
Formylglycine	(12)	" (11)	570		0
Formylphenylalanine	As for formylglycine	" (11)	570		0

* The numbers in the parentheses refer to the bibliography.

employed and the change in optical density corresponding to the hydrolysis of 0.1 μ m per ml. with various compounds are given in Table I.

Properties and Purification of Formylase

About 100 μ m of formylanthranilic acid can be hydrolyzed per hour per gm. of liver of the several species tested (dog, guinea pig, rat, rabbit, pig, and pigeon). Other rabbit tissues in which tryptophan oxidation to

kynurenine has not been observed and which will also hydrolyze formylanthranilic acid at a small fraction of the rate with liver, kidney, spleen, and intestine. Heart muscle, skeletal muscle, brain, and lung show no activity.

The relative activity of the crude enzyme of rat liver on different substrates does not differ significantly from that shown in Table I for the purified enzyme. Acetylamino compounds are hydrolyzed so poorly by the enzyme that the designation formylase seems justified. It can be seen that rapid hydrolysis is obtained only with aromatic compounds that are substituted with a formamino group ortho to an acidic group. The apparent preference of the enzyme for a compound like formylkynurenine is confirmed by the rapid hydrolysis of the synthetic compound. The closed ring compounds, isatin, acetonyl-3-hydroxy oxindole, and dioxindole, are not acted upon by either the purified or the crude enzyme.

Purification was pressed only so far as necessary to confirm that this pattern of specificity was referable to one enzyme and to provide a catalase-free preparation sufficiently concentrated for use as a reagent in the isolation of formylkynurenine and the study of the mechanism of the tryptophan oxidation. A 50 per cent yield of the activity in crude rat liver preparations, with a 35- to 50-fold increase in activity per mg. of protein, can be obtained by the following steps: removal of inactive protein by denaturation at 60° for 5 minutes, fractionation with ammonium sulfate between 50 and 70 per cent saturation at 0°, dialysis against distilled water, and adsorption of alumina gel C γ and elution with cold 0.2 M phosphate, pH 7.5. The purified preparations are stable for months either lyophilized or stored at ice box temperatures.

Cyanide, sulfide, and fluoride have no appreciable effect on the action of formylase in concentrations sufficient to inhibit the over-all conversion of tryptophan to kynurenine.

Preparation and Identification of Formylkynurenine

Witkop and Graser have reported that ozonolysis of tryptophan yields a substance which could not be crystallized but from which kynurenine could be recovered following prolonged acid hydrolysis (3). Ozonolysis of tryptophan in formamide for 10 to 15 minutes at room temperature gives a product with an absorption curve in water showing a slight peak at 315 to 320 m μ superimposed on an end-absorption curve. When purified formylase is added to this solution, there is a decrease at 315 m μ and a simultaneous increase in absorption at 360 m μ , accompanied by the formation of kynurenine. If it is assumed that the decrease at 315 m μ represented enzymatic hydrolysis of formylkynurenine to kynurenine, absorbing at 360 m μ , only a small fraction (less than 15 per cent) of the ozonolysis product consists of formylkynurenine.

Purification of the enzyme-hydrolyzable material confirmed this view. No satisfactory method for a clean separation of the large amount of inactive material has been devised, and the formylkynurenine tends to decompose during purification. The bulk of the material can be precipitated from the reaction mixture by the addition of a little alcohol and a large volume of ether. Solution in water, followed by fractional precipitation with acetone, and adsorption on and elution from Amberlite IRA-400 give substantial purification at each step, in terms of the per cent

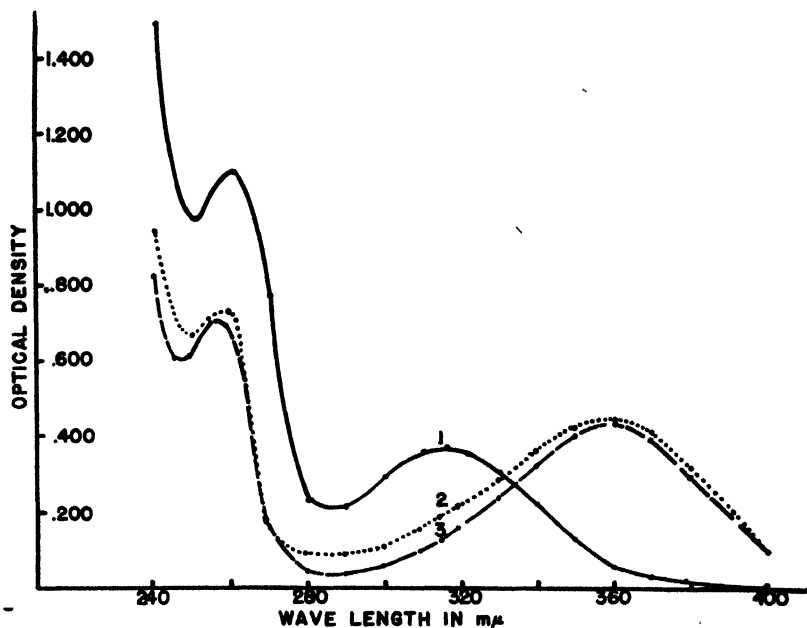


FIG. 2. Absorption spectra of kynurenine and formylkynurenine. Curve 1, formylkynurenine; Curve 2, formylkynurenine + formylase; Curve 3, kynurenine. Concentration of each 10^{-4} M, pH 7.5; depth of cell 1 cm.

converted to kynurenine upon addition of an excess of formylase. With increasing purification the absorption spectrum maximum at 315 $m\mu$ emerges more prominently. The best fraction obtained, however, came from the small amount left in the ether filtrate after the initial precipitation. On only one occasion was it possible to obtain crystals from this alcohol-formamide solution after evaporation of the ether. These formed needles on standing at room temperature and were washed with cold alcohol-ether and dried. They decompose slowly above 170° without melting. The crystalline material is very soluble in water and progressively less soluble in ethanol, acetone, and butanol.

The absorption curve of the crystalline formylkynurenine in water

shows the maximum at 315 $m\mu$ with almost complete absence of any absorption at 360 $m\mu$ due to kynurenine (Fig. 2). This maximum disappears during 10 minute hydrolysis in 1 N HCl at 100° and kynurenine is formed. Formylase likewise converts the compound quantitatively to kynurenine and formic acid, and this hydrolysis can be followed by the change in the absorption spectrum, as illustrated in Fig. 2. 7 ml. of the formylkynurenine solution used for the experiment in Fig. 2 gave rise to 7 μM of kynurenine upon addition of formylase, together with 9 μM of formic acid, determined by the method of Grant (4). The rate of this

TABLE II

Partial Separation at pH 5.4 of Coupled Tryptophan Oxidation System (Precipitate) from Formylase (Supernatant)

Kynurenine formed in 1 hour by 0.5 ml. of insoluble fraction of rabbit liver at pH 5.4 (P. 5.4) in Experiment 1, by the fraction of this P. 5.4 insoluble in 20 per cent ethanol (volume per volume; -5°) in Experiments 2 and 3, and by 2 ml. of the soluble fraction at pH 5.4 (S. 5.4). 0.05 ml. of purified formylase and 0.1 ml. of 1 per cent catalase added as indicated to the system which contained D-amino acid oxidase, DL-alanine, and 0.02 M phosphate buffer, pH 7.0; total volume 4.5 ml. as in (1). The formylase activities of P. 5.4 and S. 5.4 determined separately are given in parentheses as μM of formylanthranilate hydrolyzed per ml. per hour.

Experiment No.	S.* 5.4	P.* 5.4 + S. 5.4	P. 5.4 + catalase	P. 5.4 + catalase + formylase
	μM	μM	μM	μM
	0.05 (5.1)	2.11	0.83 (0)	1.83
	0.05 (6.5)	1.96	0.75 (0.5)	1.29
	0.05 (8.8)	2.20	0.85 (1.1)	1.68

* S. = supernatant; P. = precipitate.

hydrolysis by formylase is 6 times that of the next fastest compound, formylanthranilic acid (Table I).

*Identification of Formylkynurenine As Intermediate in
Oxidation of Tryptophan to Kynurenine*

The liver enzyme preparations able to produce kynurenine from tryptophan can be separated into two fractions by precipitating the coupled oxidation system at pH 5.4 and washing out the formylase, as described in Paper I (1). The low kynurenine formation which then occurs in the precipitated oxidizing system plus catalase can be increased by addition of the supernatant fraction or purified formylase, as shown in Table II. Since the oxygen uptake due to tryptophan is the same with or without the addition of formylase, an intermediate must accumulate during the reaction in the absence of added formylase. The absorption curve of such a reaction mixture is shown in Fig. 3. It contains some kynurenine

due to the traces of formylase persisting in the precipitated and washed oxidizing system. The curve of the intermediate is obtained by subtracting the absorption due to kynurenine and is seen to fit that of the synthetic formylkynurenine. The accumulated mixture of formylkynurenine and

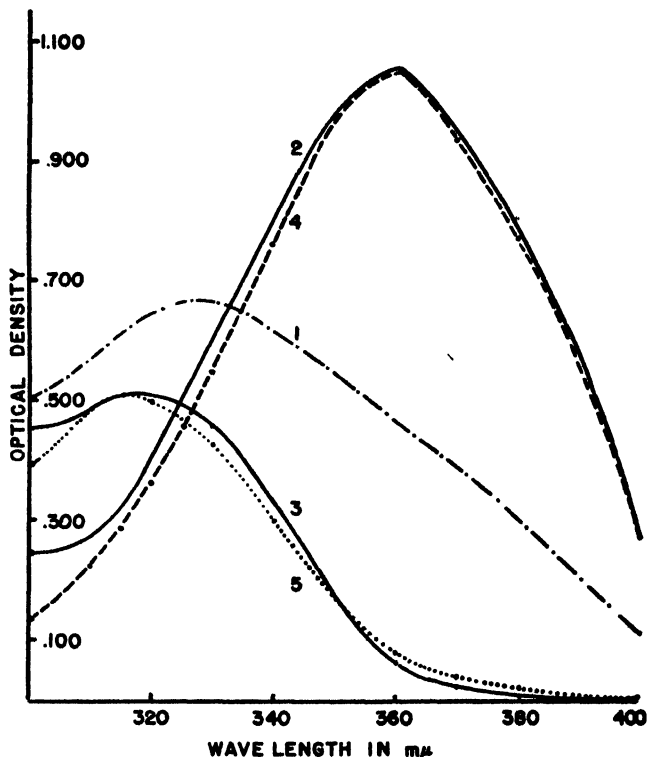


FIG. 3. Accumulation of formylkynurenine during tryptophan oxidation. Curve 1, original filtrate from tryptophan oxidation in the absence of formylase; Curve 2, original filtrate + formylase; Curve 3, Curve 1 minus kynurenine absorption; Curve 4, kynurenine; Curve 5, formylkynurenine.

kynurenine is converted to kynurenine upon addition of formylase. The deviations at low wave-lengths from the standard curves are caused by the absorption of unoxidized tryptophan in the reaction mixture.

DISCUSSION

The specificity of formylase makes it unlikely that it has previously been known by another action. It apparently contains no dissociable factor and is not inhibited by substances which interfere with hydrolytic enzymes which require metal cofactors. The relative rates of reaction of this enzyme with various substrates indicate that formylkynurenine

is probably its natural substrate. The relatively large yields of formylase in the various purification steps and the similar rates of reaction on various substrates by both crude and purified enzymes indicate the existence of a single enzyme in liver which carries out this hydrolysis. The nature of the enzymes responsible for the activity observed in tissues other than liver has not been investigated.

The very rapid hydrolysis of the compound isolated to formic acid and kynurenine by formylase after ozonolysis of tryptophan is evidence that the compound is indeed formylkynurenine. The nature of its absorption spectra before and after hydrolysis and the yield of kynurenine obtained suggest that the crystalline formylkynurenine described is of at least moderate purity. It can be characterized by its absorption maximum near 315 μ , which disappears on hydrolysis.

This absorption spectrum and its reaction with formylase have been sufficient to identify formylkynurenine as a product of tryptophan ozonolysis, as a substrate of formylase, and as an intermediate in the metabolism of tryptophan. Further chemical characterization of this new amino acid metabolite will of course be desirable.

SUMMARY

An enzyme, formylase, which hydrolyzes formylkynurenine, has been identified and partially purified; its specificity was determined and its function in the conversion of tryptophan to kynurenine established.

Formylkynurenine has been identified after ozonolysis of tryptophan and shown to be identical with the intermediate which accumulates in the absence of formylase during oxidation of tryptophan by liver enzymes.

This work was greatly facilitated by the assistance of Mr. Alexander Winterstein in the synthesis of many of the compounds tested and by the opportunity to use Dr. Byron Riegel's ozone generator.

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THE SCOPE OF THE TRANSAMINATION REACTION IN ANIMAL TISSUES*

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The transamination reaction, discovered by Braunstein and Kritzman (1), was reported early by these authors to be a general reaction involving all α -amino acids, but later was stated to be of more limited scope (2, 3). Studies by one of us, using more specific analytical techniques, indicated a limited scope to this reaction (4, 5) involving chiefly the amino acids alanine, glutamic acid, and aspartic acid. However, evidence for transamination of other amino acids, though not unequivocal, has appeared in the literature (2, 3). The high degree of specificity obtained with purified glutamic-oxalacetic and glutamic-pyruvic transaminases (6) has suggested that, unless the analytical methods previously employed were completely unreliable, there probably exist more transaminases than the aforementioned two. Tanenbaum and Shemin (7), using N^{15} -labeled substrate, reported unequivocal evidence for a leucine transaminase system. Wood and Gunsalus (8), using dried *Escherichia coli* organisms, also have recently reported transaminase activity involving a large number of amino acids.

With the availability of more reliable analytical methods, in addition to the knowledge of the coenzyme requirement, it seemed desirable to reinvestigate the scope of the transamination reaction in animal tissues. Results reported in this paper clearly establish the wide scope of this reaction.

Methods and Procedures

Enzyme Preparations—Organs were obtained fresh at the slaughterhouse and packed in ice during transportation. One part of the trimmed tissue was ground by means of a hand meat grinder (the nut butter cutter) and stirred with 1 part of cold distilled water for 5 hours in the cold (4°). The coarse material was then removed by filtering through cheese-cloth. The filtrate was centrifuged at 4000 r.p.m. for 15 minutes in a refrigerated centrifuge and the supernatant solutions were collected and lyophilized. The lyophilized preparations were stored in a vacuum at 4°.

The material designated as lyophilized homogenate was prepared by mixing equal parts of rabbit liver and 0.01 M phosphate buffer, pH 7.4, and homogenizing in the Waring blender. The homogenate was then

* Aided in part by a grant from the Wisconsin Alumni Research Foundation.

lyophilized and stored as above. Previous to use, the enzyme solutions were dialyzed in the cold (4°) against 0.01 M phosphate buffer, pH 7.4, for 12 to 18 hours.

Substrates— α -Ketoglutaric acid, prepared according to the method of Friedman and Kosower (9) with modifications, gave a yield of 50 per cent of the theoretical; m.p., 113.5–115°, as compared to the 109–110° reported (9). Details for the modified procedure will be published elsewhere. Immediately prior to use, the acid was dissolved in 0.05 M phosphate buffer, pH 7.4, at a concentration of 40 μ M per ml., unless otherwise designated. It was necessary to add sodium hydroxide to these solutions to bring them to pH 7.4.

The amino acids and peptides were used as supplied by the manufacturer, without further purification. Solutions of amino acids were made in 0.05 M phosphate buffer, pH 7.4, at a concentration of 20 μ M per ml. with respect to the L form, unless otherwise designated. The peptides were dissolved in water at a concentration of 125 μ M per ml.

Coenzyme—Pyridoxal phosphate, as the calcium salt, was supplied by W. W. Umbreit of the Merck Institute for Therapeutic Research, to whom we are greatly indebted for a generous supply. An aqueous solution at a concentration of 30 γ per ml. was used.

Glutamic Acid Decarboxylase Preparation—*Clostridium welchii*, strain S. R. 12, obtained from the American Type Culture Collection, Washington, was grown according to the method of Gale (10) and stored in the lyophilized condition. This material retained full activity for a period greater than 6 months when stored in this condition. Immediately prior to use, a suspension of 25 mg. per ml. of the lyophilized organism was prepared in 0.10 M acetate buffer, pH 5.0.

Incubation Conditions and Determination of Glutamic Acid—Transamination was measured by determining the amount of glutamic acid formed in an incubation mixture containing α -ketoglutaric acid, the amino acid in question, pyridoxal phosphate, enzyme solution, and buffer, which was made up as follows. To the main compartment of a Warburg flask was added 1 ml. of an enzyme solution which had been dialyzed for 24 hours, immediately prior to the assay, against 0.01 M phosphate buffer, pH 7.4. 0.5 ml. of pyridoxal phosphate solution was then added and the mixture allowed to equilibrate for 20 minutes at room temperature. (No attempt was made to establish saturation levels of pyridoxal phosphate in these systems.) Amino acid solution, 1 ml., was added to the flask which was then mounted in a Warburg bath by means of a special rack. After equilibration at 38°, 0.5 ml. of α -ketoglutaric acid solution was added, the flask stoppered, and the reaction allowed to proceed for 1 hour, unless otherwise indicated. The reaction was stopped by detaching the flask from the

rack and immersing it in a boiling water bath, $\frac{1}{4}$ inch deep, for a period of 5 minutes. The solution in the flask was then brought to pH 5.0 by the addition of 0.2 ml. of 3 M acetate buffer, pH 4.8, and 0.5 ml. of the suspension of *C. welchii* was added to the side arm. Glutamic acid was determined as described by Krebs (11). A blank containing all components except amino acid was routinely employed after preliminary determinations had established the fact that the organism contained no decarboxylase activity against the amino acid and no transaminase activity with the amino acid, α -ketoglutaric acid, pyridoxal phosphate incubation system. CO_2 evolution was measured until no more gas was evolved. This usually required 30 minutes under the conditions employed. Air was used as the gas phase after it was noted that O_2 uptake was negligible.

Determination of α -Ketoglutaric Acid— α -Ketoglutaric acid was determined by means of a chromatographic procedure described by LePage (12). We are greatly indebted to Dr. LePage for making the details of his method available to us prior to its publication. Hydrazones of α -ketoglutaric acid and the keto acid formed by transamination were adsorbed on a column of Hyflo Super-Cel and then eluted with mixtures of ether and alcohol which had been saturated with an acid solution. LePage has demonstrated that the hydrazones of α -ketoglutaric, pyruvic, and oxalacetic acids can be resolved on a column of diatomaceous earth. Under his conditions, we have found that the hydrazones of keto acids formed from leucine, isoleucine, tryptophan, phenylalanine, valine, tyrosine, methionine, and cysteine can be resolved from the hydrazone of α -ketoglutaric acid. In all cases, α -ketoglutaric hydrazone was adsorbed in a thin band at the top of the column and was the last to be eluted. The bands of the hydrazones of the other keto acids appeared 1 to 2 cm. below that of α -ketoglutaric acid and fell in positions which roughly correspond to those occupied by the hydrazones of pyruvic and oxalacetic acids.

α -Ketoglutaric acid was determined by extracting the eluate with aqueous alkali and the concentration determined colorimetrically in the Beckman spectrophotometer. The other keto acids were not measured quantitatively in this study, owing to the lack of pure samples. The identity of the band assayed as α -ketoglutaric acid hydrazone was established by acidifying the alkaline solution used for assay purposes, extracting with ether, combining this extract with an ether solution of the hydrazone of α -ketoglutaric acid, and passing this mixture through a column. Failure to resolve this mixture under these conditions was taken as proof that the band assayed was that of α -ketoglutaric acid hydrazone.

The hydrazones of keto acids derived from arginine and histidine did not form bands on the column, owing possibly to the relative insolubility of these hydrazones under the conditions of the extraction.

Determination of Amino Acids—Total amino acids in the incubation mixture were determined by a modification of the method of Moore and Stein (13), according to the experimental conditions suggested by R. W. McGilvery of this laboratory. To 0.5 ml. of the incubation mixture was added 1 ml. of 4 per cent perchloric acid. The precipitated protein was removed by centrifugation, and 1 ml. of the supernatant was neutralized with 1 ml. of 0.5 M dibasic sodium phosphate. To 0.1 ml. of this neutralized supernatant was added 1 ml. of ninhydrin solution, prepared according to the directions of Moore and Stein. The mixture was shaken and then heated for 20 minutes in boiling water, after which it was diluted to 10 ml. with 50 per cent alcohol, shaken, and read in the Coleman junior spectrophotometer at 570 m μ . The solutions used to construct the standard curve were run simultaneously with the unknowns. Leucine plus an enzyme preparation, treated in the same manner as the incubation mixtures, was used to construct the standard curve.

Since the incubation mixture contains two amino acids, and since it is well known that the ninhydrin method does not give the same amount of color per mole of different amino acids, it was necessary to employ the following formulation, which we derived, in order to correct for the disparity. Moore and Stein have tabulated color values which are the ratios of densities of equimolar concentrations of the amino acid in question to leucine. If we designate the color value for the ratio of colors from glutamic acid and leucine by the symbol V , then $V = k_1C_1/k_2C_2$, where k_1 and k_2 are *apparent extinction coefficients* for glutamic acid and leucine respectively, and C_1 and C_2 are their molar concentrations. We have called these coefficients *apparent extinction coefficients*, because it is known that the color is due to the same end-product in every case. Then, since $C_1 = C_2$, $Vk_2 = k_1$. Similarly, for any other amino acid $V_3k_2 = k_3$, where k_2 has the same significance as above, k_3 is the *apparent extinction coefficient* for the other amino acid, and V_3 is the color value of this amino acid. Since, in a mixture of independently absorbing compounds, the observed density may be represented as the sum of the densities of the individual components, we can write for any pair of amino acids, $D = k_1C_1 + k_3C_3$, where D is the observed density for the mixture. Substituting the values of k_1 and k_3 derived above and rearranging, we obtain $C_3 = D - Vk_2C_1/V_3k_2$. The apparent extinction coefficient, k_2 , of leucine is computed from the standard curve, while the color values V and V_3 are taken from the table of Moore and Stein. The concentration of glutamic acid, C_1 , is determined by the decarboxylase method described above.

Measurement of Peptidase Activity—Peptidase activity was measured by the methods of Grassmann and Hyde (14), according to conditions outlined by H. T. Hanson of this laboratory. In order to inhibit the action

of leucylglycine peptidase, lyophilized pig heart extract was dissolved in 0.2 M sodium citrate and was dialyzed for 24 hours against 0.02 M sodium citrate (15). Dialysis in the presence of citrate removed metal activators as a result of dialysis and complex formation with citrate ion. Reaction systems were made up in 25 ml. Erlenmeyer flasks (see legend, Table IV, for conditions). All components, other than the peptide, were added to the flasks, which were then equilibrated at 38°. The solution of the peptide was then added, and the initial blank value was determined by immediately withdrawing a 0.2 ml. aliquot and transferring it to a 10 ml. Erlenmeyer flask. Enzymatic activity was halted by placing the flask in a boiling water bath for 5 minutes. After 30 and 60 minutes aliquots were withdrawn to determine peptidase activity. At the end of 60 minutes, the 25 ml. Erlenmeyer flask was placed in a boiling water bath for 5 minutes, and a 2 ml. aliquot was withdrawn to determine transaminase activity as described above.

Results

The results of a survey of transaminase activity in various tissues are presented in Table I. It is evident that many more amino acids participate in the transamination reaction than had heretofore been realized. That this activity is not the peculiar attribute of a particular type of tissue is evident from the results for heart muscle, liver, and kidney. No emphasis is to be placed on the relative activity of a given amino acid in different tissues, since the tissue extracts were prepared simultaneously and then used at different periods of time. It became apparent later on in the study that the age of these preparations was a significant factor.

Aspartic acid appeared to be transaminated completely in muscle extract. On further study, this proved to be an error due to two facts not originally recognized. The first is that the *C. welchii* preparation possesses a glutamic-oxalacetic transaminase which is active at pH 5.0. The second is that the strain of *C. welchii* employed in these studies is capable of decarboxylating aspartic acid. The latter fact was first called to our attention by Dr. A. Meister of the National Cancer Institute. Similar observations have been recently reported by Müller and Leuthardt (16). These limitations do not apply to the other systems, since the blanks used in our studies indicated that *C. welchii* is not capable of decarboxylating any other amino acid studied, nor does it possess a transaminase capable of effecting reaction between these amino acids and α -ketoglutaric acid at pH 5.0. Suitable assay methods for studying the kinetics of the glutamic-oxalacetic system, as well as an alcohol fractionation scheme for the preparation of a highly purified glutamic-oxalacetic transaminase, will be the subject of another communication from this laboratory.

The slight activity exhibited by β -alanine, taurine, and the D-amino acids can be ascribed to L-amino acid impurities which are capable of participating in the transamination reaction. In general, a value of less than 5 μ l.

TABLE I
Transaminase Activity in Tissue Extracts

	Heart muscle		Liver		Kidney	
	CO ₂ measured	Per cent trans-aminated	CO ₂ measured	Per cent trans-aminated	CO ₂ measured	Per cent trans-aminated
	μ l.		μ l.		μ l.	
L-Aspartic acid	449	100	265	70	381	89
DL-Valine	237	53	65	14	37	9
L-Leucine	246	54	99	22	123	27
DL-Isoleucine	208	51	47	10	27	6
L-Alanine	170	38			61	14
L-Tyrosine	82	18	144	32	67	15
L-Phenylalanine	68	15	73	16	27	6
DL-Methionine	57	13	55	12	12	2
L-Tryptophan	49	11	17	4	21	5
L-Arginine	29	7	132	28	144	31
L-Cysteine	28	6	46	10	2	0.5
DL-Serine (40 μ M)	13	1.4	-7	0	-3	0
DL-Histidine (40 μ M)	10	1.2	-6	0	5	0.5
Glycine (40 μ M)	8	0.8	57	6.4	-2	0
DL-Threonine (40 μ M)	2	0.2	-5	0	3	0.3
DL-Lysine (40 μ M)	13	1.4	4	0.4	12	1
L-Cystine (40 ")	3	0.2	-18	0	-7	0

Heart muscle					
L-Citrulline (50 μ M)	11.4	1	β -Alanine	10	3
L-Ornithine (50 ")	75.7	8	Taurine	7	12.5
3,4-Diiodo-L-tyrosine	34.5	8	DL-Norleucine	75	16
DL-Dopa	25	6	L-Methyltyrosine	85	22
DL- α -Amino-n-butyric acid	40	10	D-Phenylalanine	7	1.5
α -Aminoisobutyric "	6	1	D-Leucine	9	1.6

The reaction mixtures contained 20 μ M of α -ketoglutaric acid, 100 mg. of lyophilized, dialyzed tissue extract, 15 γ of pyridoxal phosphate, and 20 μ M of amino acid, unless otherwise indicated, in a total volume of 3 ml. Incubation time 1 hour; pH 7.4.

of CO₂ is not considered significant, since the organisms alone exhibited a negative uptake value of approximately 3.0 μ l. of O₂, and it was felt that the total of 8 μ l. of gas was within the normal range of experimental error. No attempt was made to determine whether the methyl ester of tyrosine was active as a result of hydrolysis of the ester to tyrosine.

After completing the survey, it was necessary to demonstrate that the reaction observed was due to transamination. Proof of the nature of the reaction took two routes: first, by the exclusion of other possible mechanisms, and secondly, by the demonstration that the stoichiometric balance of reactants and products was in accordance with the proposed reaction.

One possible mechanism for the formation of glutamic acid was the reductive amination of α -ketoglutaric acid first reported by Dewan (17) and by von Euler *et al.* (18). Since reductive amination is necessarily coupled with an oxidative process, it was conceivable that this reaction could be coupled with the oxidative decarboxylation of α -ketoglutaric acid reported by Krebs and Cohen (19). As can be seen from Table II, ammonia plus α -ketoglutaric acid did not result in the formation of any more CO_2 than might be expected to arise from the blank. Indeed, a pronounced inhibi-

TABLE II
Effect of Ammonia on Formation of Glutamic Acid and Transamination

Reactants	Isoleucine		Tryptophan		Methionine		Ammonia		Blank	
	-	+	-	+	-	+	+	+		
Ammonia	-	+	-	+	-	+	+	+		
CO_2 formed, $\mu\text{l.}$	207	193	89	64	61	61	13	11	13	14
Change, %		-6.8		-28		0				

The reaction systems contained 20 μM of amino acid, 20 μM of α -ketoglutaric acid, 15 γ of pyridoxal phosphate, 100 mg. of lyophilized, dialyzed heart muscle extract, and, where indicated, 20 μM of ammonia in a total volume of 3 ml. Incubation time 1 hour; pH 7.4.

tory effect is apparent, and, accordingly, it is felt that this mode of formation of glutamic acid can be excluded.

The second method of proof that these observations are indeed due to a transamination reaction is the balance study presented in Table III. If one starts with 40 μM of amino acid and 40 μM of α -ketoglutaric acid, it would be expected that if the reaction is really one of transamination, then the concentration of added amino acid and α -ketoglutaric acid at the termination of the reaction should be the same. As can be seen from Columns 1 and 2 of Table III, this expectation has been realized. Also, one would expect that, if α -ketoglutaric acid were stoichiometrically converted to glutamic acid, then the total number of moles of these two substances at the termination of the reaction should equal the number of moles of α -ketoglutaric acid at the start of the reaction. As can be seen from Column 4, this expectation has also been realized, and, accordingly, it seems safe to assume that the observations reported here are due to transamination.

The totals of Column 4 (Table III) seem to differ systematically from

the expected total of 40 μM . This difference, however, may be more apparent than real. The analytical methods employed are certainly not more accurate than 3 to 5 per cent, and, since the methods employed were micro-methods, requiring large dilution factors to bring the data up to the absolute figures quoted, the average deviation of 1.5 μM from the expected figure of 40 μM represents an error which is within the experimental limits, even if the deviations are not scattered as might be expected.

TABLE III
Transaminase Balance Study

Amino acid	Added amino acid present at end of incubation (1)	Added α -ketoglutaric acid present at end of incubation (2)	Glutamic acid formed (3)	Added α -ketoglutaric acid accounted for ((2) + (3)) (4)
	μM	μM	μM	μM
Isoleucine...	12.5	16.1	21.6	37.7
Arginine...	29.6	26.6	11.4	38.0
Tryptophan...	16.5	16.6	21.3	37.9
Phenylalanine	20.3	22.2	16.2	38.4
Valine.....	20.1	19.7	19.4	39.1
Histidine....	32.0	34.6	2.5	37.1
Tyrosine.....	20.4	19.5	19.0	38.5
Leucine.....	19.0	24.0	21.4	45.4
Methionine.....	15.7	21.0	16.0	37.0
Cysteine.....	27.6	31.0	4.7	35.7

The reaction systems contained 40 μM of the L form of amino acid, 40 μM of α -ketoglutaric acid, 30 γ of pyridoxal phosphate, 300 mg. of lyophilized, dialyzed heart muscle extract in a total volume of 6 ml. Incubation time 3 hours; pH 7.4.

It has been previously demonstrated that pyridoxal phosphate is the coenzyme required for transamination in the glutamic-oxalacetic and the glutamic-pyruvic systems (8, 20, 21), and, accordingly, it seemed of interest to determine what effect this substance would have on the systems reported here. Amino acids were selected from both ends of the activity scale and tested with and without pyridoxal phosphate. As is evident from Table IV, pyridoxal phosphate does have an activating effect on all the systems studied, and, since these systems were selected at random, it probably has an activating effect on all the amino acids presented in Table I. Table IV also illustrates the fact that the preparations used in this study represent partially resolved enzymes. Thus it seems that previous failures to demonstrate the existence of many transaminases were due not only to inadequate analytical procedures but also to the low activity of most of the transaminases, as a result of partial resolution of the enzyme and its coenzyme.

Past studies on transaminase activity have emphasized the stability of these enzymes, but our experience with an alcohol-fractionated, highly purified glutamic-oxalacetic transaminase¹ suggests that these enzymes are unstable. Green *et al.* (6) have shown that the glutamic-oxalacetic tran-

TABLE IV
Effect of Pyridoxal Phosphate

Reactant	Leucine		Isoleucine		Tyrosine		Tryptophan		Methionine	
	+	-	+	-	+	-	+	-	+	-
Pyridoxal phosphate	+	-	+	-	+	-	+	-	+	-
CO ₂ formed, μ l.	240	174	210	141	144	130	113	73	68	43
Activation, %	38		48		11		55		58	

The reaction systems contained 20 μ M of the L form of amino acid, 20 μ M of α -keto-glutaric acid, 100 mg. of lyophilized, dialyzed heart muscle extract, and, where indicated, 15 γ of pyridoxal phosphate in a volume of 3 ml. Incubation time, 1 hour; pH 7.4.

TABLE V
Effect of Age on Transaminase Activity

Amino acid	Age of preparation		Inactivation
	2 wks.	8 wks.	
Lyophilized muscle extract			
	μ l. CO ₂	μ l. CO ₂	per cent
Methionine.....	57	50	0
Valine.....	237	64	73
Isoleucine.....	208	84	60
Phenylalanine.....	67	71	0
Tryptophan.....	65	60	0
Lyophilized liver extract			
Cysteine.....	46	8	82
Glycine.....	57	9	84
Arginine.....	147	119	19
Tyrosine.....	144	78	46
Leucine.....	99	37	63

The reaction systems contained 100 mg. of lyophilized, dialyzed extract, 20 μ M of the L form of amino acid, 20 μ M of α -ketoglutaric acid, 15 γ of pyridoxal phosphate, all in a total volume of 3 ml. Incubation time, 1 hour; pH 7.4.

saminase, when in solution and in the presence of ammonium ion, is unstable. Our purified preparation, which was stored in the lyophilized state, in the cold, and in an atmosphere of nitrogen, lost four-fifths of its activity after a period of 4 weeks. The effects of age on two different

¹ Cammarata, P. S., and Cohen, P. P., unpublished data.

preparations are presented in Table V. It is apparent that, even in our crude preparations, in which protective influences might be expected in accordance with the observation that an increase in purity usually leads to a decrease in stability, the enzyme deteriorates with age, even if stored in the lyophilized state, *in vacuo*, and in the cold.

Although no absolute quantitative significance can be placed on the individual values for a given amino acid in Table I, it is believed that the patterns of activities in the three different tissues are significant in that the different ratios of activities indicate that they are due to a number of enzymes rather than to a single one. This belief is confirmed by the differential activation of these enzymes, illustrated in Table IV, and the differential inactivation, illustrated in Table V. This belief is further confirmed by as yet unpublished studies on the specificities of a highly purified glutamic-oxalacetic transaminase¹ and a highly purified glutamic-pyruvic transaminase,² both of which were tested with the technique presented above. Neither of these purified enzyme preparations exhibited activity against any of the amino acids other than the specific amino acid for which they had been fractionated. Since many of these amino acids can be considered as derivatives of alanine, the lack of activity with the purified glutamic-pyruvic enzyme is especially significant.

The present studies are concerned with the transaminase activity found in the soluble protein fraction of tissues. There is reason to believe that other transaminases may exist in the residue of tissue extracts. Thus, with a lyophilized whole homogenate of rabbit liver, rather potent serine and histidine transaminases were revealed, whereas no such activity was demonstrated in the pig liver extract. It is realized that the use of liver from a different animal has introduced a new variable, and, accordingly, studies concerned with the intracellular distribution of the different transaminases are being conducted.

The rôle of peptides in transamination has been suggested by Linderstrøm-Lang (22) and investigated by Ågren (23). While the latter reported evidence for a transamination reaction between valylglycine and α -ketoglutaric acid, the analytic method employed makes the validity of this claim open to question. In view of the possible importance of such a reaction, it was decided to reinvestigate the rôle of peptides in transamination. For this purpose, three peptides were chosen for study. Two of these, leucylglycine and alanylglycine, contained constituent amino acids capable of transaminating with α -ketoglutaric acid, while the third, glycylglycine, contained as its constituent an amino acid of limited activity in the system studied. Leucylglycine was selected because its hydrolysis could be inhibited almost quantitatively by removal of the metal ion acti-

² Rand, M. C., and Cohen, P. P., unpublished data.

vator, under mild conditions which would not inactivate the enzymes involved (15). As a control on the treatment of the enzyme preparation, leucine was included in the study.

In preliminary experiments with leucylglycine and an enzyme preparation which had not been freed of metal ion activator, this peptide apparently transaminated to α -ketoglutaric acid. It is evident from Table VI, however, that there is a positive correlation between the degree of hydrolysis and the extent of transamination, and that transamination from these peptides does not occur under the conditions employed, unless hydrolysis precedes the transamination reaction. Leucylglycine, which was not hydrolyzed, did not transaminate. Alanylglycine, which was hydrolyzed, produced the amount of CO_2 expected from alanine alone, and glycylgly-

TABLE VI
Transamination Studies Utilizing Peptides

Reactant	Leucylglycine		Alanylglycine		Glycylglycine		Leucine
	-	+	-	+	-	+	+
α -Ketoglutaric acid	-	+	-	+	-	+	+
Hydrolysis in 60 min., %	10	7	87	90	50	54	
CO_2 formed in 60 min, μl .	2	51	17	700	13	32	91
Theoretical CO_2 , μl .	0	1600	0	1600	0	1600	224
Transamination, %	0	3.2	0	42.7	0	2	37.5

The reaction systems contained 20 mg. of lyophilized, dialyzed pig heart extract, 15 γ of pyridoxal phosphate, 125 μM of α -ketoglutaric acid, 0.5 ml. of citrate phosphate buffer (0.1 M sodium citrate in 0.05 M phosphate buffer, pH 7.4), 125 μM of peptide, and 20 μM of leucine. Incubation time, 1 hour; pH 7.4.

cine, which was only 50 per cent hydrolyzed, yielded practically no CO_2 . The leucine control produced sufficient CO_2 to indicate that the transaminase systems were active under the conditions employed.

An attempt to demonstrate the participation of methylglyoxal in a transamination reaction with glutamic acid gave negative results. Accordingly, although one can hardly generalize on the basis of a relatively few experiments with a limited number of members of two classes of compounds, it seems likely that the transaminase reactions which we have observed are restricted to reactions between α -keto acids and α -amino acids. Our experiments, while demonstrating the scope of this reaction with regard to the amino acid component of the system, do not give any indication of the ability to substitute other keto acids for α -ketoglutaric acid.

DISCUSSION

In the present study certain shortcomings of previous investigations, now apparent in retrospect, have been overcome. The use of extracts,

homogenates, or minced preparations of tissues in the early studies (1-3) did not permit concentration of the enzyme preparation necessary for demonstrating the presence of the weaker transaminases. The use of lyophilized extracts in the present study, on the other hand, has made this possible. Thus a lyophilized extract can be dissolved to give a concentration which is 30 or more times greater than that obtained with a homogenate or mince made up of 1 part of original tissue plus 3 parts of buffer. A second factor which was not appreciated in the earlier studies was that of the need for added coenzyme. A third factor, which aided in the recognition of the wide scope of the transamination reaction, was dialysis of the enzyme solutions. In preliminary experiments it was found that the blank values of solutions of undialyzed muscle, liver, and kidney extracts were as high as 150 μ l. of CO_2 . Following dialysis, the blank value of the liver and kidney preparations was reduced to approximately 50 μ l. of CO_2 , while the blank value of the muscle extract was completely eliminated. The high blank values of the tissue preparations used in the earlier studies made difficult the assessment of the significance of small changes in glutamic acid formation. Dialysis resulted in a reduction of the glutamic acid blank value to such an extent that the significance of increases in glutamic acid in the experimental incubation systems could be readily evaluated. Thus, the recognition of these three factors together with the use of more accurate analytical methods has made it possible to demonstrate the wide scope of the transamination reaction.

The demonstration from this study that the transamination reaction is of wide scope, including possibly all of the natural amino acids, gives this reaction a unique rôle in intermediary metabolism. In addition to the ready interconversion of the key metabolites, oxalacetic, α -ketoglutaric, and pyruvic acids to aspartic acid, glutamic acid, and alanine, respectively, transamination must now be recognized as being of importance in oxidative deamination and amino acid synthesis. Braunstein and Azarkh (24) reported early the coupling of transaminating enzymes with glutamic dehydrogenase with the resulting formation of ammonia from the added amino acids. If such a coupling reaction proves to be a general one for all the amino acids, then the failure to date to demonstrate a specific L-amino acid oxidase of sufficient activity to explain the metabolism of L-amino acids in tissue slices is understandable. On the basis of a reversal of the transamination reaction, one can also explain the synthesis of amino acids from corresponding α -keto acids.

□ While the data obtained with peptides would suggest that these are not involved in transamination reactions, the possible importance of this reaction in peptide synthesis would make it highly desirable to investigate the activity of keto peptides. It is possible that the equilibrium conditions

are more favorable for the reverse reaction. In this connection it is noteworthy that Herbst and Shemin (25) observed non-enzymatic transamination of pyruvylalanine.

In view of recent observations that the amino group of aspartic acid can transaminate with citrulline to form arginine in liver (26, 27), it is clear that one-half of the nitrogen in urea can arise from amino acids without the necessity for the latter to undergo oxidative deamination. Additional metabolic interrelationships involving transamination reactions have been reviewed by Braunstein (3).

SUMMARY

1. Twenty-two amino acids, in addition to alanine, aspartic acid, and glutamic acid, have been shown to participate in the transamination reaction. The reaction is catalyzed by aqueous extracts of pig heart, liver, and kidney. Each transamination reaction appears to be due to a different transaminase.

2. The reaction is accelerated by pyridoxal phosphate and slightly inhibited by ammonia.

3. Three peptides and methylglyoxal have been shown not to participate in the reaction under the conditions employed.

4. New methods, which combine simplicity and a high degree of specificity, have been developed for the study of transaminating systems.

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IDENTIFICATION OF L-ASCORBIC ACID IN THE URINE OF NORMAL AND CHLORETONE-TREATED RATS*

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About 10 years ago, King, Longenecker, and their associates made the interesting observation that the urine of rats subsisting on a milk diet which was supplemented with a non-saponifiable fraction from oat oil, grass leaf oil, or alfalfa leaf oil (2) or with certain terpenes (3) or hypnotics, particularly chloretone (4), contained considerable quantities of a reducing substance. This reducing substance could be oxidized rapidly by 2, 6-dichlorophenolindophenol. Urine from rats treated with the non-saponifiable material from oats (2), or with *d*-carvone, isophorone (3), or phenobarbital (4), had an antiscorbutic potency for guinea pigs corresponding approximately to the ascorbic acid content of the urine as measured by indophenol titration. Inasmuch as no other naturally occurring substance is known to have an equally high antiscorbutic potency, the reducing substance in the urine was presumably L-ascorbic acid. A number of other investigators have confirmed the effect of chloretone on the increase of reducing substances in the urine of rats (5-8). Cattle (9-11) and sheep (12) have been reported to give a similar response to chloretone administration.

Preliminary to other studies in this laboratory it was considered desirable to isolate and characterize the principal reducing substance present in the urine of chloretone-treated rats. At the same time a procedure was worked out for the isolation, in pure form, of a 2, 4-dinitrophenylhydrazine derivative of L-dehydroascorbic acid from materials containing relatively small quantities of ascorbic acid. Inasmuch as the precise structures of this derivative and its possible isomers (13) are not known, as well as for brevity of presentation, this derivative will be referred to subsequently in this paper as Compound I. The isopropylidene derivative prepared from Compound I will be referred to as Compound II.

EXPERIMENTAL

Isolation of L-Ascorbic Acid—Urine was collected from mature male rats fed evaporated milk and having access to water. The rats were kept in

* Paper No. 2565, Scientific Journal Series, Minnesota Agricultural Experiment Station. This study was aided by a grant from Swift and Company, Chicago, Illinois. A preliminary report of some phases of this work was presented at the meetings of the American Society of Biological Chemists, at Detroit, 1949 (1).

metabolism cages and the urine was collected in a solution of 6 per cent oxalic acid. The pooled urines were stored in a frozen condition until used. Increased ascorbic acid excretion by the rats was induced by daily intraperitoneal injections of 21.4 mg. of chloretone (1,1,1-trichloro-2-methyl-2-propanol) dissolved in 0.1 ml. of corn oil. During the isolation procedure the concentration of ascorbic acid in the various fractions was followed by titration with 2,6-dichlorophenolindophenol. A typical isolation experiment involved the following steps: Pooled urine was filtered through Hyflo Super-Cel (Johns-Manville) and 970 ml. of the clear filtrate were treated with solid CaCO_3 until the pH was 5.1. The solution was cooled in an ice bath, 145 ml. of a 20 per cent solution of mercuric acetate were then added, and the solution was allowed to stand for 5 minutes. (All ascorbic acid was oxidized during this step.) To this suspension, 170 ml. of a 20 per cent solution of lead acetate were added. After filtration through Super-Cel the greenish solution was decolorized by addition of 25 gm. of charcoal (Darco G-60) and filtered. The filtrate was treated with H_2S for 15 minutes to precipitate mercury and lead and to reduce dehydroascorbic acid. The excess H_2S was then removed *in vacuo* under nitrogen. At this point titration with indophenol indicated an 81.5 per cent recovery of the reducing substances originally present in the urine. To the cold solution, 80 ml. of a 20 per cent solution of lead acetate were added. The solution turned yellow-brown but was decolorized by filtration through Super-Cel. The lead salts were precipitated by addition of 10 per cent NH_4OH to pH 7.4, filtered after cooling for 30 minutes, and washed with cold water. Judging from the reducing substances remaining in the solution and washings, the lead salts should have contained 60 per cent of the reducing material originally present in the urine. The lead salts were washed with acetone, then suspended in 150 ml. of acetone, and decomposed with H_2S ; 5 gm. of charcoal (Darco G-60) were added and treatment with H_2S was continued for 5 minutes. After filtration through Super-Cel the solution was concentrated *in vacuo* under nitrogen to 3 ml., when droplets of an immiscible liquid formed.¹ The concentrate was treated with 12 ml. of *n*-butanol and 0.5 ml. of ethanol and warmed to 50° under a stream of nitrogen. Addition of a total of 30 ml. of petroleum ether (b.p. 40–45°) in several portions over a period of 5 days and cooling at -18° caused the separation of a syrupy material on the sides and bottom of the vessel. After decantation of the supernatant the syrup was taken up in 1 ml. of methanol. The addition of 2 ml. of benzene yielded a two-phase system

¹ If human urine, to which ascorbic acid has been added in quantities equivalent to that found in the urine of chloretone-treated rats, is carried through the same procedure, the addition at this stage of 2 volumes of benzene to the concentrate results in crystallization of ascorbic acid upon cooling. With rat urine crystallization could never be induced without further purification.

from which white crystals separated after prolonged cooling at -18° . The crystals were washed three times with 1 ml. of cold (-10°) ethanol. The compound was recrystallized from *n*-butanol with the addition of petroleum ether and cooling at -18° . The crystalline material was finally washed with petroleum ether and dried *in vacuo* at 50° . The yield of recrystallized ascorbic acid in this experiment was 75.9 mg. Based on the indophenol titration of the original rat urine, calculated in terms of ascorbic acid, the recrystallized compound was isolated in about 10 per cent yield. The greatest loss occurred in the final steps, after decomposition of the lead salts.

The identity of the recrystallized compound, isolated from urine, with L-ascorbic acid is shown by the following data (corresponding values for recrystallized synthetic L-ascorbic acid are given in parentheses): m.p., 191° ² (191°); mixed melting point 191° ; $[\alpha]_D^{21}$ in methanol, $+50.5^{\circ}$; concentration, 13.2 mg. per ml. ($+50.8$ (14)); reducing value, 174 mg. were equivalent to 1 mm of I_2 (176 mg.). For further identification the compound was converted into 5,6-isopropylidene-L-ascorbic acid (15) which had a melting point of 220° ($220-222^{\circ}$), a mixed melting point of 220° , and a reducing equivalent of 228 mg. per mm of I_2 (theoretical 216 mg.). The slightly decreased reducing value of the isopropylidene derivative may be due to some oxidation of the compound during its preparation.

Isolation of Compound I from Urine—For studies on the biosynthesis of ascorbic acid its isolation in pure form did not appear practical because of the low yield obtained. Other investigators (16, 17) have isolated from human urine a 2,4-dinitrophenylhydrazine (DNPH) derivative of dehydroascorbic acid by methods involving chromatographic purification with Al_2O_3 as adsorbent. The formation of this derivative also is the basis of the widely used method of ascorbic acid analysis described by Roe and Kuether (18).

Preliminary experiments showed that chromatography on Al_2O_3 of the crude mixture of DNPH derivatives prepared from rat urine was not promising for the isolation of Compound I in good yield and in a high degree of purity. Of various adsorbents examined a mixture of 3 parts of $Mg_3(PO_4)_2 \cdot 4H_2O$ (Baker's analyzed grade) and 1 part of Hyflo Super-Cel (Johns-Manville) permitted satisfactory separation of the desired compound. The crude DNPH derivatives were prepared from rat urine (collected in 6 per cent HPO_3) by oxidation of the filtered urine with 2,6-dichlorophenolindophenol, followed by the addition of thiourea and DNPH in 9 N H_2SO_4 as suggested by Roe and Kuether (18). To increase the yield of the desired derivative the mixture was incubated at 50° for 6 hours. The precipitate was then filtered with the aid of Super-Cel, thoroughly washed with dilute acid and water, and dried. The DNPH derivatives

² Melting points, uncorrected, were determined with the Fisher-Johns apparatus.

were dissolved in absolute acetone (purified by refluxing with KMnO_4 , drying over K_2CO_3 , and distilling). Aliquots of these solutions were used for chromatography in columns of about 8×50 mm. The chromatogram was developed with absolute acetone. A yellow fraction passed rapidly through the column, followed by several slower moving fractions which separated into distinct zones upon development of the column with abso-

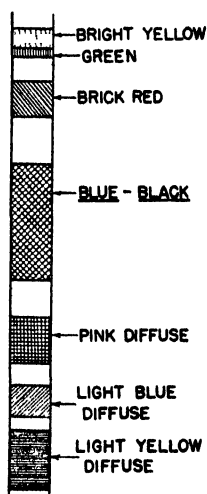


FIG. 1

FIG. 1. Typical appearance of developed chromatogram of urinary DNPH derivatives on magnesium phosphate tetrahydrate.

FIG. 2. Absorption spectra of DNPH derivatives from normal rat urine dissolved in 12.5 M H_2SO_4 .

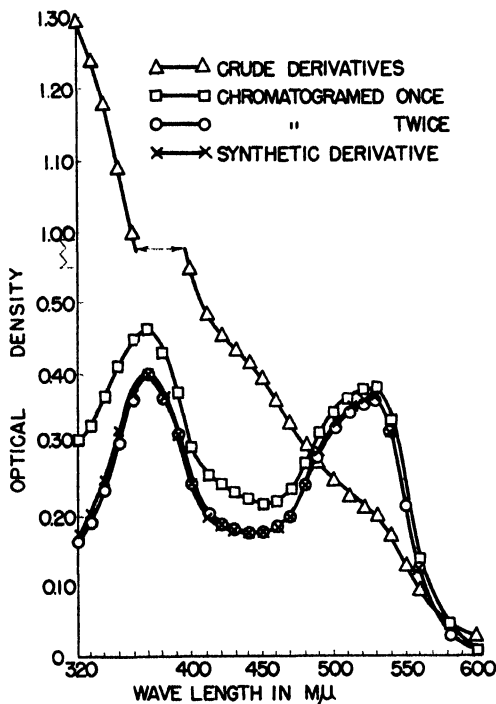


FIG. 2

lute acetone, as shown in Fig. 1. Compound I comprised the blue-black zone. Upon prolonged development with acetone, a green fraction trailed immediately behind the blue-black fraction. This green fraction appeared to be closely related to Compound I, since it yielded an identical absorption spectrum in 12.5 M H_2SO_4 and since yellow acetone solutions of the synthetic Compound I (prepared from pure L-ascorbic acid) slowly became green when treated with MgCO_3 in the presence of traces of water. Com-

pound I could be recovered from the column by prolonged development with acetone, as well as by mechanical separation of the extruded adsorbent followed by elution with 0.1 M oxalic acid in absolute acetone. A second chromatographic purification of the desired fraction usually yielded a product having spectral properties identical with those of synthetic specimens of Compound I. Prior to a second chromatographic purification it is necessary to remove the oxalic acid which was used in the eluant. Of several different procedures tried for this purpose, the following gave the best results for quantitative work: The acetone-oxalic acid eluate was evaporated to about 3 ml. and diluted with 10 ml. of water, and the precipitate was filtered and washed with water. The precipitate was then dried and dissolved in acetone for further chromatographic purification or for crystallization from 1:1 acetone-ethanol.

Properties of Compound I

Absorption Spectra—As a guide in the chromatographic purification of this compound we have used the absorption spectrum of its solution in 12.5 M H_2SO_4 . (All absorption spectra were measured with the Beckman spectrophotometer, model DU.) The extinction coefficients $E_{1\text{cm}}^{1\%}$ for Compound I dissolved in 12.5 M H_2SO_4 were found to be 520 at λ 530 $\text{m}\mu$, 549 at λ 370 $\text{m}\mu$, and 242 at λ 450 $\text{m}\mu$.³ In the mixture of crude DNPH derivatives isolated from the urine of normal rats the characteristic maxima and minima of Compound I observed in the spectral region of 320 to 600 $\text{m}\mu$, as shown in Fig. 2, are masked by the absorption of other compounds. After one chromatographic purification, however, or with DNPH derivatives prepared from the urine of chloretone- or carvone-treated rats, absorption curves were obtained which resemble those given by the synthetic Compound I. A second chromatographic separation yielded a compound which showed the same absorption spectrum as a synthetic standard (Fig. 2). The ratios of the optical densities measured at the wave-lengths 530:450, 370:450, and 370:530 $\text{m}\mu$ appear to be an excellent criterion of the purity and identity of the DNPH derivative of ascorbic acid (Table I). The absorption spectrum in 0.1 N NaOH can be used as a confirmatory criterion of identity (Fig. 3). Spectrophotometric measurements of Compound I in 0.1 N NaOH should be made within 1 hour after the preparation of the solution, since the alkaline spectrum of Compound I changes gradually. It should be pointed out, in addition, that the absorption spectrum in sodium hydroxide shows marked shifts, depending upon the alkalinity of the solution. Furthermore, different isomers of Compound I which may be formed during chromatography on magnesium phosphate

³ Reexamination of the extinction coefficients $E_{1\text{cm}}^{1\%}$ reported previously revealed that they were in error and should be replaced by the values given here (1).

tetrahydrate have different extinction coefficients at the absorption maxima of 420 and 530 $m\mu$ when dissolved in dilute sodium hydroxide. If an acetone solution of these isomers is treated with a trace of oxalic acid, however, the isomers are converted to the same form, which has the absorption spectrum in 12.5 M H_2SO_4 shown in Fig. 2.

Melting Point—There is no agreement in the literature on the melting point of Compound I. Inasmuch as values ranging from 258–290° have been reported by various investigators (16, 17, 19–24), the melting point is of questionable value as a criterion of identity and purity. Herbert *et al.*

TABLE I
Ratios of Optical Densities of Urinary DNPH Derivatives in 12.5 M H_2SO_4

Diet of rats	Treatment of rats	Ratios of optical densities			Chromatographic purification
		530 $m\mu$ 450 $m\mu$	370 $m\mu$ 450 $m\mu$	370 $m\mu$ 530 $m\mu$	
Evaporated milk	None	0.508	2.26	4.45	None
“ “	“	1.76	2.13	1.21	Once
“ “	“	2.02	2.29	1.13	Twice
“ “	Chloretone	1.36	2.16	1.59	None
“ “	“	2.06	2.32	1.14	Once
Stock ration*	None	0.560	2.29	4.09	None
“ “	“	1.72	2.29	1.33	Once
“ “	“	2.03	2.31	1.14	Twice
“ “	Chloretone	1.48	2.30	1.55	None
“ “	“	2.01	2.36	1.17	Once
Synthetic reference standard†		2.05 ± 0.04	2.26 ± 0.03	1.09 ± 0.02	

* Commercial grain ration containing some animal products.

† The values for the reference standards are the means and standard deviations of eight synthetic specimens of Compound I. Six of these were recrystallized from a 1:1 acetone-ethanol mixture, and two were chromatographed and then crystallized from acetone-ethanol.

(19) have pointed out that there are numerous possibilities for structural isomerism and stereoisomerism among the phenylhydrazine derivatives of L-dehydroascorbic acid. By recrystallization from pyridine, from acetic acid, or from acetone-ethanol we have always obtained the crystalline Compound I, melting with decomposition at 291–292°. This applies to synthetic specimens and to those isolated from the urine of normal and chloretone-treated rats.

Quantitative Isolation of Compound I—Two 50 ml. portions of normal rat urine were used. Each contained 2.55 mg. of ascorbic acid as determined by the method of Bessey (25). To one portion, 10 mg. of ascorbic

acid were added. Each portion was diluted to 1000 ml. with 5 per cent metaphosphoric acid, oxidized with 2,6-dichlorophenolindophenol, and treated with 250 ml. of 1 per cent 2,4-dinitrophenylhydrazine in 9 N H_2SO_4 containing 0.25 gm. of thiourea per 100 ml. (18). To obtain a maximum yield of Compound I the mixture was incubated for 7 days at 38°. The precipitates were then filtered, washed, extracted with acetone, and chromatographed as described before. After a second chromatographic purification of the fraction containing Compound I the absorption spectrum of its solution in 12.5 M H_2SO_4 indicated that it was essentially pure. Based on the extinction coefficient at 530 $\text{m}\mu$ in 12.5 M H_2SO_4 , the concentration of Compound I was calculated. From the portion of rat urine containing

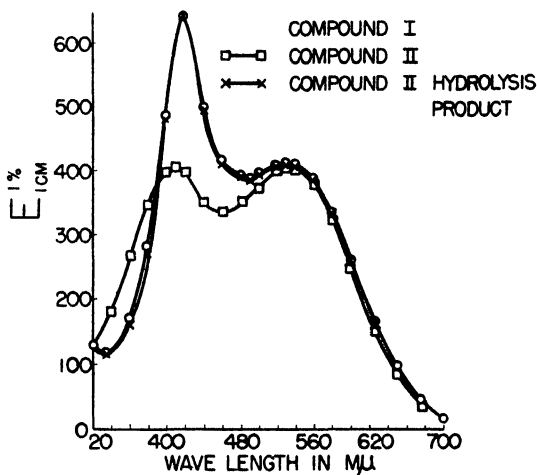


FIG. 3. Absorption spectra of DNPH derivatives in 0.103 N NaOH

originally 2.55 mg. of ascorbic acid, 7.6 mg. of Compound I, equivalent to 2.48 mg. of ascorbic acid, were isolated after purification by two chromatographic adsorptions. From the other portion, containing 2.55 mg. plus 10 mg. of added L-ascorbic acid, 37.4 mg. of Compound I, equivalent to 12.30 mg. of ascorbic acid, were isolated.

Preparation and Properties of Compound II—For further identification of Compound I, isolated from the urine of normal and chloretone-treated rats, this compound was converted into its 5,6-isopropylidene derivative and compared with a synthetic specimen. The preparation of the isopropylidene derivative can be readily carried out by the following adaptation of the procedure used by Engel (26) for the preparation of 5,6-isopropylidene-D-galactosazone: 20 mg. of Compound I were dissolved in 5 ml. of redistilled *p*-dioxane and 10 ml. of absolute acetone. After the

compound had dissolved, the temperature of the solution was maintained at 30° while a total of 0.2 gm. of P_2O_5 was added in small increments and with mechanical stirring during 1 hour. Stirring was then continued for 2 hours. The solution was filtered, washed with saturated aqueous K_2CO_3 , and the aqueous layer was discarded. The filtered solution was treated with a few drops of 0.1 M oxalic acid in absolute acetone until the color of the solution became orange-red, filtered again, and then evaporated to dryness *in vacuo*. The residue was recrystallized from a 1:1 mixture of acetone and ethanol. The yield was 90 per cent of the theoretical. The synthetic Compound II and that prepared from the urine of normal and chloretone-treated rats had a melting point of 275°. Mixed melting points of synthetic and natural derivatives showed no depression. Elementary analysis of the synthetic compound gave C 43.74, 43.95, N 19.40, 19.54, H 3.34, 3.34 (calculated for $C_{21}H_{18}N_3O_{12}$, C 43.90, N 19.50, H 3.16 per cent).⁴

By boiling with 90 per cent acetic acid, prepared from repeatedly recrystallized glacial acetic acid, Compound II described above was quantitatively hydrolyzed to yield acetone and Compound I. The latter, m.p. 289–291°, was recovered from the acetic acid solution either by direct crystallization or by dilution with water and recrystallization from acetone-ethanol. The acetone formed during hydrolysis was distilled into a cooled receiver and quantitatively determined by the iodoform reaction according to Pregl (27). Thus from 29.9 mg. of Compound II 3.095 mg. of acetone were obtained (calculated for the monoacetone derivative, 3.025 mg.). Fig. 3 illustrates the absorption spectra in dilute sodium hydroxide of Compound II and of its product of hydrolysis. The good yields obtained in the formation and subsequent hydrolysis of the Compound II suggest that it may serve a useful purpose for the purification of Compound I after a preliminary chromatographic separation of the latter.

SUMMARY

1. From the urine of chloretone-treated rats, L-ascorbic acid has been isolated in pure form.
2. A procedure has been described for the isolation of a pure 2,4-dinitrophenylhydrazine derivative of L-dehydroascorbic acid from the urine of normal rats. The chromatographic adsorption used for this purpose permits quantitative recoveries under suitable conditions.
3. The preparation of the 2,4-dinitrophenylhydrazine derivative of 5,6-isopropylidene-L-dehydroascorbic acid has been described.

⁴ Elementary analyses for C and H by Mr. R. Kelly, Department of Organic Chemistry, University of Minnesota; for N by the Clark Microanalytical Laboratory, Urbana, Illinois.

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THE MALTASE OF *CLOSTRIDIUM ACETOBUTYLICUM*

ITS SPECIFICITY RANGE AND MODE OF ACTION*

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The starch-splitting enzyme system of *Clostridium acetobutylicum* has attracted occasional attention and been of some general interest due to the ability of this system to break down starch entirely to glucose, neither maltose nor limit dextrins being found to any appreciable extent in the conversion products. This ability to convert starch to glucose has been shown by Hockenhull and Herbert (1) to be due to the presence of two distinct enzymes: an amylase of the α type which rapidly dextrinizes the starch and converts it approximately to maltose, together with a maltase which further hydrolyzes the maltose to glucose.

Hockenhull and Herbert in commenting on the substrate specificity of *C. acetobutylicum* maltase stated, without giving experimental details, that it is a "true" maltase, *i.e.*, specific for maltose and without action on α -glucosides in general (*e.g.*, sucrose). In the present study we have examined the action of this maltase on amylooligosaccharides, such as amylo-triose, amyloheptaose, isomaltose, and other saccharides which one might reasonably expect to occur as intermediate or final products of α -amylase action on starch. In addition a number of substances including α -methyl glucoside, glucose-1-phosphate, sucrose, raffinose, cellobiose, starch, limit dextrins, and the Schardinger dextrins were investigated in order to check the conclusions of Hockenhull and Herbert and extend if possible the limitations on the specificity of this maltase.

EXPERIMENTAL

Enzyme Preparation—A soil culture of *Clostridium acetobutylicum* was obtained from Dr. L. A. Underkofler of the Chemistry Department, Iowa State College. It was grown on sterile corn-meal medium at 37–38°. The 40 hour corn-meal (20 ml.) culture was inoculated into 2 liters of sterile yeast extract-maltose medium (1 per cent Difco yeast extract, 2 per cent

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maltose, 5 per cent calcium carbonate) and held at 30–32° approximately 60 hours. The culture fluid was then filtered through a Horm laboratory pressure filter,¹ concentrated to about 70 ml. by evaporation in an air stream, dialyzed for 24 hours against distilled water, filtered, and stored under toluene in the refrigerator. The enzyme so obtained was stable over a period of several months. It had a maltase activity of 0.5 unit per ml., expressed in terms of the Hockenhull and Herbert maltase unit.² The amylase activity was appreciable, even when dextrin-free maltose was used in the culture medium.

In order to remove amylase from the enzyme preparations, they were starch-adsorbed in the cold as follows: To 100 ml. of cold enzyme solution were added 20 ml. of cold acetone and 20 gm. of acetone-extracted potato starch (1, 2). The suspension was stirred at 0° for 20 minutes and filtered, and the acetone removed by vacuum distillation in the cold. Enzyme solutions so obtained had appreciably lower maltase activity (0.2 maltase unit per ml.); however, there was no detectable trace of amylase activity following the acetone-starch treatment.

While the enzyme activity was rather low in these preparations, it was found to be sufficient to hydrolyze 10 mg. of maltose per ml. of enzyme solution essentially quantitatively in 2 to 3 days, or 75 mg. of maltose per ml. of enzyme solution to about 90 per cent in a similar period. In other experiments the enzyme retained activity over a period of 3 to 4 weeks at 37°, and thus the low activity could be compensated for by using prolonged digestion times. It would be highly desirable to obtain a richer source of enzyme; however, no exploratory work was done along this line.

Substrates Used—C.p. maltose hydrate, Merck, was found by paper chromatography (3) to contain appreciable amounts of trisaccharide and higher dextrans. In some cases, carefully purified, dextrin-free maltose was used without changing the experimental results significantly. The Schar-dinger dextrans and amyloheptaose were prepared in this laboratory by published procedures (4, 5). The limit dextrans were prepared by extended enzyme action on potato starch, followed by alcohol precipitation of the limit dextrin. The isomaltose sample was kindly furnished by Dr. Edna Montgomery, and the dextran by Dr. Allene Jeanes, both of the Northern Regional Research Laboratory, Peoria, Illinois. On examination by paper chromatography the isomaltose appeared to contain a substantial amount of the homologous trisaccharide and other saccharides; the effect of these contaminants was carefully considered in reaching conclusions on the action of maltase on isomaltose.

¹ Manufactured by F. R. Hormann and Company, Brooklyn, New York.

² 1 unit hydrolyzes 5 mg. of maltose in 1 hour at 37° (0.5 per cent maltose, pH 4.25).

The other substrates used were standard laboratory samples.

Enzyme Digests—Initially the digests were made up in the proportion of 40 ml. of enzyme solution, 10 ml. of acetate buffer of pH 4.5, and 50 ml. of 6 per cent substrate solution (final substrate concentration, 3 per cent). Due to the relatively low activity of the enzyme it was found more advantageous to drop the substrate concentration to 0.5 per cent, as used by Hockenhull and Herbert (4.5 ml. of enzyme solution, 1.5 ml. of acetate buffer, and 3.0 ml. of 1.5 per cent substrate solution). The enzyme digests were held at 37° in a constant temperature incubator; enzymatic action was stopped by heating to 100° or by the addition of the reaction aliquots to the copper reagents.

Methods of Analysis—Glucose was determined quantitatively by a spectrophotometric adaptation of the colorimetric micromethod of Tauber and Kleiner (6), standardized by the use of mixtures of glucose with the substrates under investigation. A linear relationship was found relating the optical density (Coleman universal spectrophotometer) at 650 m μ and the amount of glucose present. The total reducing value was determined by the Swanson and Cori modification (7) of the Shaffer and Somogyi alkaline copper method (8), or by a colorimetric procedure exactly analogous to the Tauber and Kleiner glucose determination except that the Shaffer and Somogyi reagent No. 60, made up without iodide or iodate, was used with a 30 minute boiling period in place of the acid copper reagent with an 8 minute boiling period. Qualitative evaluation of the composition of enzyme digests was obtained by paper chromatography as described previously (3). The linear amylooligosaccharides were found to fall into a regular series, the first eight being clearly resolved; on plotting the log R_f values (9) against the molecular size a straight line was obtained which includes all except the lowest members of the series (Fig. 1). Isomaltose was found to fall between maltose and amylotriose; other saccharides containing a single 1-6 linkage appeared to fall into positions approximately half way between the corresponding linear saccharide and the next higher linear saccharide. Oligosaccharides of the dextran series, produced by partial acid hydrolysis of dextran, were separated considerably more from each other than corresponding amylooligosaccharides; for example the dextran trisaccharide had about the same R_f value as amylotetraose.

RESULTS AND DISCUSSION

Purity of Enzyme—While Hockenhull and Herbert state that they were able to prepare an amylase-free maltase by culture of the bacteria on maltose, in our studies such cultures always contained considerable amylase, even when media from c.p. and carefully recrystallized maltose were inoculated from cultures on c.p. maltose. Therefore, in order to obtain a

maltase preparation as free from amylase as possible, the crude enzyme was subjected to starch adsorption; such a procedure had been used by Hocken-hull and Herbert in order to concentrate the amylase and we found it equally effective in removing amylase from the maltase preparations. As an indication of the freedom of our preparations from amylase, we ascer-tained that the characteristic blue iodine color remained through the major part of the conversion of starch to glucose; at about 80 per cent conver-sion the blue reaction shifted toward purple and, as hydrolysis became nearly complete, the iodine color became red and finally colorless. It is

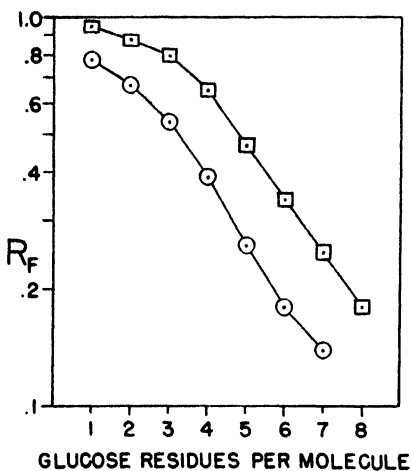


Fig. 1

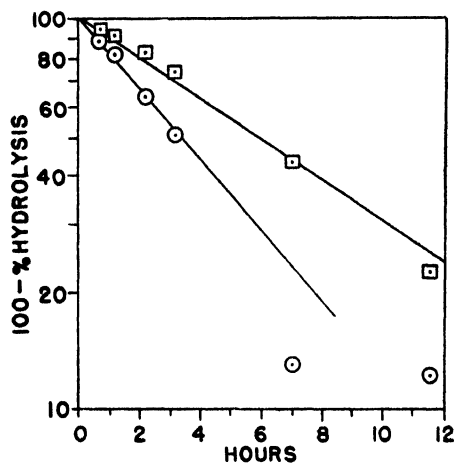


Fig. 2

FIG. 1. Resolution of amylooligosaccharides on multiple ascent paper chromatograms. (○) four ascents; (□) twelve ascents. The apparent R_F values represent the fraction of the total height of the paper climbed by the individual saccharides after four and twelve ascents, respectively. The ordinate (R_F) is plotted on a logarithmic scale.

FIG. 2. Rate of action of maltase on maltose (○) and amyloheptaose (□). The ordinate is plotted on a logarithmic scale. Substrate concentration, 0.5 per cent.

fairly obvious that it would be possible to assay these maltase preparations for amylase activity by the viscometric, iodine color, or reducing value methods but the significance would be questionable. Inasmuch as the iodine color reaction undergoes a pronounced shift between 10 and 20 per cent total hydrolysis with ordinary α type amylases, we have interpreted the persistence of the blue iodine reaction to at least 80 per cent hydrolysis as an indication of the virtual freedom of our maltase preparations from amylases of the α type. β -Amylases can be excluded on the grounds that the sole low molecular weight product of hydrolysis is glucose, as judged

by the equivalence of the amount of glucose formed with the total increase in reducing power, together with the observation of glucose as the only detectable reducing sugar when examined by paper chromatography. From the foregoing it appears that the enzyme is reasonably free from amylase and that such traces of amylase as may be present should in no way affect the validity of the main conclusions of this paper.

Kinetics of Enzyme Action—Glucose determinations at intervals during the digestion of maltose and amyloheptaose indicated that the kinetics obeyed only approximately the first order law over the portion of the curve up through 70 to 80 per cent hydrolysis (Fig. 2). No significant differences were observed in the rates of glucose formation by given amounts of the enzyme at 0.5 and 3.0 per cent maltose concentrations. If the K_m value indicated by Hockenhull and Herbert (0.0016 mole per liter) is of the right order of magnitude,³ the concentrations used by us were sufficiently high to insure maximal reaction rate. The deviation from the first order kinetics is in the direction of zero order, as one would expect.

While it would be advantageous to investigate more carefully the enzyme kinetics, in this study it was our primary concern to obtain qualitative rather than quantitative information regarding the specificity and action pattern of the enzyme. For this purpose, it was convenient to assume that the relative reaction rates for the enzyme with various substrates could be adequately expressed as the apparent first order constants under essentially identical digestion conditions. The results of the digests for several of the substrates investigated are collected in Table I.

Action on Maltose—In confirmation of the work of Hockenhull and Herbert, the only reducing sugar produced from maltose was glucose. While the activity of the enzyme was so low that 2 or 3 days were required for completion of the hydrolytic action, in each case the conversion proceeded to the extent of 100 per cent within the limit of the experimental error (about 3 per cent with the Tauber and Kleiner micromethod, spectrophotometrically).

Action on Amyloheptaose—The rate constant given in Table I for amyloheptaose is just over half that for maltose. In consideration of the fact that there are only about half as many glucosidic bonds per glucose residue

³ The experimental determination of the K_m for *C. acetobutylicum* amylase given by Hockenhull and Herbert appears to be in error in that the enzyme digests at low substrate concentrations proceeded to virtually complete hydrolysis; from these it is impossible to determine the initial rates necessary for the determination of the enzyme-substrate dissociation constant. Since no experimental details were given for the determination of the maltose-maltase dissociation constant, one may inquire whether a similar error may have been made in the latter case also.

in maltose as there are in amyloheptaose, this relationship between rate constants indicates clearly that the *rate of cleavage of glucosidic bonds in amyloheptaose is just as fast as in maltose*. In other words, amyloheptaose is as specific a substrate for this "maltase" as is maltose itself.

The qualitative composition of amyloheptaose digests at intermediate stages of completion was ascertained by paper chromatography. In these, it was apparent that the amyloheptaose was disintegrating in a stepwise manner: at first amylohexaose appeared (together with glucose, which shortly after the beginning of the digest was the predominating component on the chromatogram); then as larger amounts of the intermediate saccharides amylotriose, amylotetraose, and amylopentaose became evident, the amyloheptaose diminished and disappeared. In the final stages, the

TABLE I
*Relative Rate Constants for Hydrolysis of Various Substrates by
C. Acetobutylicum Maltase*

Substrate, 0.5 per cent concentration	Period of observation	Apparent 1st order rate constant
	days	sec. ⁻¹
Maltose	3	6.7×10^{-6}
Amyloheptaose	3	3.4×10^{-6}
Starch	8	3.7×10^{-6}
Salivary amylase limit dextrin	9	$\sim 1 \times 10^{-6}$
β -Amylase limit dextrin	8	2.0×10^{-6}
<i>B. macerans</i> -amylase limit dextrin	8	1.9×10^{-6}
Isomaltose	28	1.0×10^{-6}
Sucrose	8	1.3×10^{-6}
Raffinose	5	$< 1 \times 10^{-7}$
Cellobiose	36	9×10^{-8}
β -Schardinger dextrin	8	$< 5 \times 10^{-8}$
γ -Schardinger "	8	$< 5 \times 10^{-8}$

only oligosaccharide remaining was maltose, and at last this disappeared entirely. It appears that one can explain these observations qualitatively by postulating a single hydrolytic attack by the enzyme on the terminal glucosidic linkage, producing glucose and the next lower homologue of the substrate; subsequently the enzyme and products separate by diffusion before the once attacked substrate is attacked again. If the next substrate molecule undergoing hydrolytic attack by the enzyme is determined by the statistical distribution of the remaining possible substrate molecules, attack on any one substrate molecule is equally probable to that on any other. This view is in distinct contrast to reaction mechanism pictures which have been developed for α - and β -amylases (10-12) in that with the latter multiple attack on the substrate seems to be the general rule.

Action on Whole Starch and Limit Dextrins—The initial rates of hydrolysis of starch, amyloheptaose, and maltose were found to be essentially identical, and whole starch gave eventually nearly complete conversion to glucose. The fact that the blue iodine color persists through the major part of the reaction, besides indicating freedom from amylase, also intimates that the hydrolytic action takes place at the terminal linkages of starch chains rather than at interior linkages. This feature of maltase action is somewhat similar to current views on the point of attack of β -amylase, and the parallelism is further reflected in the fact that the blue iodine color persists similarly throughout β -amylase action. In other respects these enzymes differ significantly; apart from the difference in reducing sugars produced, in the case of maltase the iodine color eventually disappears (here one may not rule out entirely the effect of a trace of α -amylase in the maltase preparations), and moreover β -amylase action stops far short of complete conversion to maltose, leaving a high molecular weight limit dextrin.

The ability of maltase to hydrolyze starch completely to glucose, without leaving any limit dextrin, etc., suggested the possibility that this enzyme might be capable of attacking limit dextrins formed by the action of other enzymes. This was indeed found to be the case, and by the action of maltase the limit dextrins formed by β -amylase, salivary amylase, and *Bacillus macerans* amylase were converted to glucose. It was not surprising to find that hydrolysis took place at a rather slower rate than with starch or maltose, and in each case, nearly quantitative amounts of glucose were formed.

Action on Isomaltose—Inasmuch as maltase was shown to be capable of causing extensive hydrolysis of limit dextrins, it was not surprising to find that it has a slow but definite action on compounds of the isomaltose type. The kinetics of the isomaltose digest were anomalous in that the hydrolysis proceeded at a rapid rate until about 25 per cent of the substrate had been converted to glucose. At this point the hydrolysis slowed down to less than one-tenth of the initial rate, and remained steady throughout the remainder of the digest. In order to calculate the rate constant in Table I, it was assumed that the first 25 per cent phase of the hydrolysis was due to the presence of saccharides other than isomaltose (especially isomaltose homologues, which could readily be detected by paper chromatography) and that the rate of hydrolysis characteristic for isomaltose itself was given from that point on. It is reasonable that, if the sample contained compounds in which 1 or more glucose residues were joined to the non-reducing terminus of an isomaltose moiety, these might behave very much like the terminal glucose residues in amyloheptaose or other starch oligosaccharides, thus giving a rapid initial hydrolysis.

While the main phase of action on isomaltose was very slow indeed, by allowing the digest to stand for several weeks a total conversion to glucose of over 85 per cent was obtained, with no indication that the reaction had stopped; presumably, if the digestion had been sufficiently prolonged, the total conversion would have approached 100 per cent.

Action on Sucrose and Raffinose—Although Hockenhull and Herbert state that maltase is without action on sucrose, we have repeatedly observed a definite slow hydrolysis, at about the same rate as with isomaltose. The failure by Hockenhull and Herbert to observe hydrolytic action on sucrose was most likely due to the slow rate of action together with a rather short period of observation. In order to show extensive hydrolysis of sucrose or isomaltose, we have found it necessary to extend observations over a period of several days; for example, in one experiment sucrose was hydrolyzed to the extent of 69 per cent in 8 days. In view of this apparent sluggishness with sucrose, one may well ask whether such a result might not be due to the presence in the enzyme preparation of a trace of invertase. In an attempt to answer this question raffinose has been subjected to the action of maltase under the same conditions used for sucrose. No detectable hydrolysis of raffinose resulted; we conclude thereby that the enzyme is free from fructosidase action and that most likely the hydrolysis of sucrose is produced by the same enzyme that hydrolyzes maltose. Here again one cannot rule out entirely the possibility that a specific sucrase is present. In this connection, Robinson (13) observed the hydrolysis of maltose but not of sucrose in toluene-treated suspensions of *C. acetobutylicum*. On the other hand, maltose, sucrose, and raffinose are fermented by the organism. Robinson concludes that, while the cells may produce both sucrase and maltase, the sucrase must act *intracellularly*, while the maltase may be secreted and act *extracellularly*.

Action on Other Substrates—No detectable hydrolysis took place with α -methyl glucoside, glucose-1-phosphate, or the Schardinger dextrins. Failure to act with any appreciable speed on α -methyl glucoside or the Cori ester suggests that this maltase operates best on a substrate in which glucose is joined through an α -glucosidic linkage to another carbohydrate unit, rather than to an aglucon such as methanol or phosphoric acid. In the case of the Schardinger dextrins, the cyclic structure presents nowhere a terminal glucosyl group; it is therefore by no means possible to obtain glucose from the Schardinger dextrins by a *single* hydrolytic step. The lack of action of maltase on the Schardinger dextrin is comparable to that observed with β -amylase, which cannot hydrolyze the cyclic dextrins. On the other hand, the cycloamyloses, especially the β - and γ -dextrins, are not entirely resistant to the action of α -amylases.

The extremely slow rate of action on cellobiose is of questionable sig-

nificance and is given for the sake of record only. With dextran, after a slight initial hydrolysis (less than 0.5 per cent), no further reaction could be observed.

SUMMARY

The maltase of *C. acetobutylicum* is a glucosidase having as its principal catalytic activity the hydrolytic removal of individual glucose units from the non-reducing terminus of a starch chain or starch oligosaccharide. On encountering a branch point in the starch structure, the enzyme is capable of hydrolyzing at least the majority of the branch linkages, resulting in eventual extensive hydrolysis of starch or open chain dextrans to glucose. The relative rate of action is given for several substrates including starch, dextrans, and oligosaccharides.

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CHEMICAL PROPERTIES OF FERRITIN AND THEIR RELATION TO ITS VASODEPRESSOR ACTIVITY*

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The studies of Granick and Michaelis (1) on the chemistry of ferritin and its iron-free protein, apoferritin, were concerned with their preparation in crystalline form, the nature of the iron in ferritin, and the rôle of ferritin as an iron storage compound for hemoglobin regeneration. Ultra-centrifuge data obtained by Rothen (2) have shown that ferritin is a mixture of some free apoferritin together with ferritin, the latter in an aggregated state. Apoferritin, however, gives a single uniform sedimenting boundary with a molecular weight for horse spleen apoferritin of 465,000.

Work in our laboratory (3) has led to the identification of ferritin with a hepatic vasodepressor material which appears in the circulation in low but measurable concentrations during the irreversible or hyporeactive stage of hemorrhagic shock in animals, and, together with a vasoexcitor material of kidney origin, in the circulation during experimental renal hypertension in dogs and essential hypertension in man.

The present study is concerned with the further exploration of some chemical properties of ferritin and apoferritin and their relation to the vasodepressor activity of these proteins as measured by the rat mesoappendix test of Zweifach and Chambers (4). Data are presented from experiments on electrophoresis, viscosity, fractionation studies with ammonium sulfate and high speed centrifugation, the effects of crystalline pepsin and trypsin, the effect of pH on the vasodepressor activity, as well as a complete amino acid distribution.

EXPERIMENTAL

Crystalline ferritin and apoferritin were prepared¹ by the method of Granick and Michaelis (5) as modified by the authors (3). Total nitrogen was determined by a modified micro-Kjeldahl method (6), total phosphorus

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by that of Fiske and Subbarow (7), total iron by the Wong method (8), total S according to Zahnd and Clarke (9), and cadmium by the method described by Granick and Michaelis (5). Amino nitrogen was determined by the Van Slyke micromethod (10) and all amino acids with the exception of tryptophan and cystine by the microbiological procedure of Henderson and Snell (11) with *Lactobacillus arabinosus*, *Leuconostoc mesenteroides*, and *Streptococcus faecalis*.² Tyrosine and tryptophan were determined by the

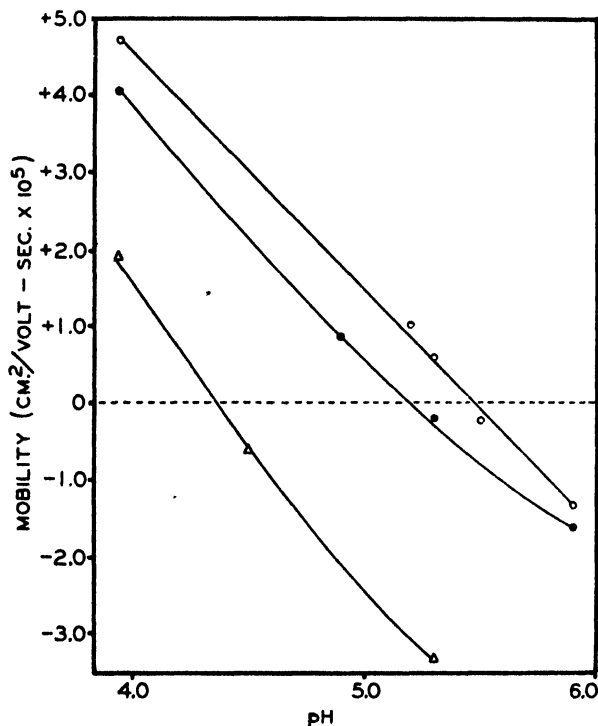


Fig. 1. Electrophoretic mobilities of horse spleen (Δ), human liver ($\circ$), and dog liver ($\bullet$) ferritins at various pH values. All determinations were done in acetate buffer, 0.1 ionic strength.

method of Folin and Marenzi (12) adapted to the photocolormeter, and total cystine by the method of Kassell and Brand (13).

Electrophoretic Behavior of Ferritin—It had been previously reported (14) that the mobilities of crystalline ferritin and apoferritin prepared from horse spleen were identical over a wide pH range, and that these proteins

² We are indebted to Dr. Ernest Borek of the New York State Psychiatric Institute for the specimen of *L. arabinosus* and to Dr. Erwin Brand of the College of Physicians and Surgeons for *L. mesenteroides* and *S. faecalis*.

gave uniform single boundaries on electrophoresis. Fig. 1 illustrates the data obtained with ferritins from horse spleen, dog liver, and human liver. Electrophoretic data were obtained by use of a Longworth-Tiselius apparatus³ with 0.2 per cent ferritin solutions dialyzed against acetate buffers of 0.1 ionic strength. All boundaries were uniform and showed no evidence of contamination with proteins of different mobilities. The isoelectric points calculated from these data were 4.4 for horse, 5.2 for dog, and 5.5 for human ferritin. These data, together with the immunochemical differences previously observed (14) among these species of ferritin, indicate dissimilarity with respect to surface-charged amino acid groupings. Sev-

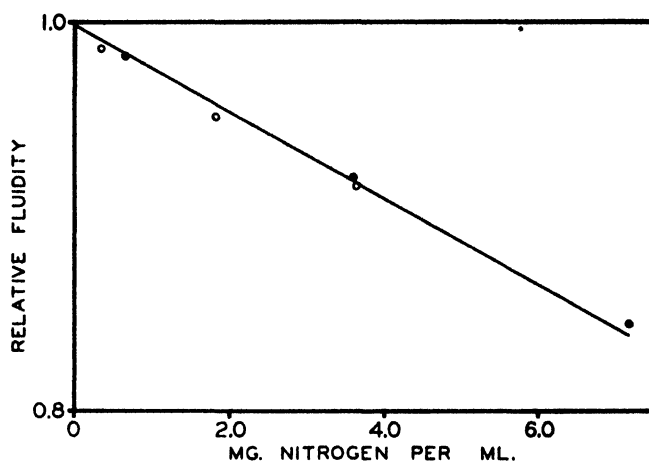


Fig. 2. Relative fluidities of horse spleen ferritin (●) and apoferritin (○) at various concentrations of protein. The determinations were done in 0.1 M phosphate buffer, pH 7.4, at 25°.

eral preliminary observations on horse liver ferritin gave mobility values identical with those shown for horse spleen ferritin. This finding would confirm our earlier observations concerning the immunochemical identity of the ferritins of horse liver and spleen.

Viscosity of Ferritin and Apoferritin—Since the viscosity of a solution is related to the volume occupied by the solute molecules as well as to their shape, it was of interest to compare ferritin with apoferritin because of the presence of much iron in ferritin and the lack of homogeneity of ferritin in the ultracentrifuge as reported by Rothen (2). Treffers (15) has observed that the relative fluidity (the reciprocal of the relative viscosity) varies as a linear function of concentration with many proteins.

³ We are indebted to Dr. V. du Vigneaud for the use of the electrophoresis apparatus.

The relative viscosity of the protein solutions was measured in a 0.1 M phosphate buffer, pH 7.4, with an Ostwald viscosimeter. Fig. 2 demonstrates that the relative fluidity of ferritin and apoferritin is identical at several different concentrations of protein N and that a linear relationship exists. This would indicate that the iron in ferritin is without effect on the viscosity owing to the protein moiety.

Fractionation of Ferritin by Ammonium Sulfate—In order to demonstrate the relationship between the iron and protein moieties of ferritin, a solution of horse spleen ferritin recrystallized four times was treated succes-

TABLE I
Fractionation of Ferritin by $(\text{NH}_4)_2\text{SO}_4$

All determinations were performed on solutions which had been thoroughly freed of $(\text{NH}_4)_2\text{SO}_4$ by dialysis. Dry weight analyses were calculated after drying aliquots of the solution to constant weight at 110° . The vasodepressor activity is expressed as the lowest concentration of protein nitrogen per 0.5 ml. which gave a positive response in the rat mesoappendix test.

Per cent saturation with $(\text{NH}_4)_2\text{SO}_4$	Per cent of original total N in ppt.	Mg. Fe Mg. N	Molar ratio, Fe:P	Analyses, dry weight basis			Vasodepressor activity $\gamma \text{ N} \times 10^{-4}$
				N	Fe	P	
				per cent	per cent	per cent	
Original	100	1.88	9	11.0	20.7	1.29	5
0 -23	18.4	2.20	10	10.2	22.5	1.27	5
23 -26	21.5	2.09	10				
26 -30	38.3	1.83	10				
30 -32.4	11.0	1.64	10				
32.4-34.6	6.0	1.46	10				
34.6-40	2.3	0.93	9	13.6	12.6	0.77	5
40 -50	0.4	0.55					5
Apoferritin*				16.2	0	0.05	5

* Apoferritin was prepared by treating the original ferritin with $\text{Na}_2\text{S}_2\text{O}_4$ in 1 M acetate buffer, pH 4.6, and dialyzing against α, α' -dipyridyl according to Granick and Michaelis (5).

sively with varying amounts of a saturated ammonium sulfate solution. The precipitated protein was freed of ammonium sulfate by dialysis and analyzed for Fe, N, and P. The results are shown in Table I. The original ferritin solution, which was homogeneous electrophoretically, contained 1.88 mg. of Fe per mg. of N. The first precipitate obtained at 23 per cent saturation with ammonium sulfate had a Fe:N ratio of 2.20, whereas the Fe:N ratio of the last fraction, precipitated at 40 to 50 per cent saturation of ammonium sulfate, was 0.55. The ratio of Fe:P was fairly constant in all fractions, bearing out the conclusions of Granick and Michaelis (5) as to the probable nature of the iron hydroxide-iron phosphate micelles.

All fractions gave identical crystals with CdSO_4 . The original solution, as well as those fractions precipitated at 0 to 23, 34.6 to 40, and 40 to 50 per cent saturation with ammonium sulfate, gave identical vasodepressor activities on a nitrogen basis when tested by the rat mesoappendix technique (4).

Fractionation of Ferritin by Centrifugation—Rothen's results with the ultracentrifuge led us to fractionate a ferritin solution at 13,000 r.p.m. for 1 hour at 4° in a Servall centrifuge. After centrifugation, an almost colorless, thin layer of solution could be observed at the top. A portion of the top layer was removed by pipette, but some mixing with the dark brown layer underneath it was unavoidable. Similarly, an intermediate portion of the solution was obtained, as well as some from the very bottom

TABLE II

Fractionation of Ferritin by Centrifugation

Horse spleen ferritin was centrifuged in 10 ml. portions at 4° for 1 hour at 13,000 r.p.m. in a Servall angle centrifuge. The vasodepressor activity is expressed as the lowest concentration of protein nitrogen per 0.5 ml. which gave a positive response in the rat mesoappendix test.

Fraction	Total N per ml.	Total Fe per ml.	Mg. Fe Mg. N	Vasode- pressor activity
	mg.	mg.		$\gamma \text{ N} \times 10^{-4}$
Original.. . . .	5.54	10.43	1.88	5
Top layer.. . . .	1.07	1.38	1.28	5
Intermediate layer .. .	5.11	10.00	1.96	5
Bottom layer	0.32	0.80	2.49	5

of the tube which had settled almost as a pellet. Analyses gave results (Table II) similar to those obtained by chemical fractionation with ammonium sulfate. Again, all fractions were equally active on a nitrogen basis by the rat mesoappendix test for vasodepressor activity.

Effect of Pepsin on Ferritin and Apoferritin—The action of crystalline pepsin on ferritin and apoferritin was followed by the method of Anson (16) on an incubation mixture containing 0.23 mg. of horse spleen ferritin N per ml. together with 0.17 mg. or 0.017 mg. of pepsin N at 35° . Digestion was measured by means of total nitrogen determinations on aliquots of the trichloroacetic acid filtrates and corrected for the appropriate enzyme controls. Table III lists the results of a time-course reaction at pH 2.2. The difference in extent of digestion between ferritin and apoferritin was marked. Because of these results, the extent of peptic hydrolysis was studied at various pH values (Table IV). Enzyme action was allowed to continue for 1 hour at 35° and horse spleen ferritin was compared with

horse spleen apoferritin and a substrate solution prepared from dog hemoglobin according to Anson. At the same time, the extent of denaturation of ferritin and apoferritin at the different reactions was measured in terms

TABLE III

Time-Course of Peptic Hydrolysis of Ferritin and Apoferritin

The method of Anson (16) was used to estimate peptic hydrolysis, with crystalline pepsin at 35°. Enzymatic action is expressed in terms of per cent of the ferritin nitrogen found in the trichloroacetic acid filtrates. The time-course of digestion was carried out at pH 2.2.

Time	Ferritin		Apoferritin	
	0.17 mg. pepsin N	0.017 mg. pepsin N	0.17 mg. pepsin N	0.017 mg. pepsin N
<i>min.</i>				
10	13	9	90	82
30			93	87
<i>hrs.</i>				
1	16	12	93	88
2	16	12		
3	17	14	98	89
4	19	14	98	93

TABLE IV

Effect of pH on Peptic Hydrolysis and Denaturation of Ferritin and Apoferritin

The extent of hydrolysis is expressed in terms of per cent of the protein nitrogen found in the trichloroacetic acid filtrates after 30 minutes incubation at 35°, corrected for controls. All proteins were present in a concentration of 0.23 mg. of N per ml., together with 0.017 mg. of pepsin N per ml. The hemoglobin solution was prepared from dog blood by the method of Anson (16). The extent of denaturation is expressed in terms of per cent of the total N insoluble at pH 4.4 (isoelectric point of ferritin) after prior incubation for 1 hour at 35° at each pH. Each precipitate was washed twice with 5 volumes of water before analysis.

pH	Ferritin		Apoferritin		Hemoglobin
	Hydrolysis	Denaturation	Hydrolysis	Denaturation	Hydrolysis
1.6	67	69	99	79	96
2.0	16	35	79	47	89
2.5	3	3	17	17	19
3.0	0	0	4	4	18
3.5	0	0	2	0	9

of the quantity of insoluble protein precipitated by bringing the pH of the solution to the isoelectric point of this species of ferritin and apoferritin, 4.4. In general, it may be seen that greater digestion takes place at lower

pH values. Also, there is an approximate correlation between the amount of digestion and the extent of denaturation.

Since ferritin, which contains the iron, is more difficult to denature than apoferritin, a comparison was made between the peptic hydrolysis of ferritin with a high iron content and of a specimen with some or all of its iron removed. The ferritin was treated with $\text{Na}_2\text{S}_2\text{O}_4$ in 1 M acetate buffer, pH 4.6, at room temperature according to the method of Granick and Michaelis (5). After 30 minutes, a portion was removed and dialyzed against running water. The remainder was again treated with $\text{Na}_2\text{S}_2\text{O}_4$ and α, α' -dipyridyl and dialyzed until free of iron, yielding a solution of apoferritin. Table V lists the Fe:N ratio of each fraction and the relative degree of digestion by pepsin. There is a good correlation between iron content and difficulty of peptic hydrolysis.

TABLE V

Effect of Iron Content on Extent of Peptic Hydrolysis of Ferritin

Fraction A was prepared from Sample 1287 (horse spleen ferritin) by treatment with $\text{Na}_2\text{S}_2\text{O}_4$ in 1 M acetate buffer, pH 4.6, for 30 minutes and dialyzed against running water. Fraction C was prepared by allowing the $\text{Na}_2\text{S}_2\text{O}_4$ to act on Sample 1287 until all the iron had been reduced and removed by dialysis. It represents apoferritin. The extent of hydrolysis at pH 2.2 and 35° is expressed in terms of per cent of the total protein N found in the trichloroacetic acid filtrates.

Ferritin sample No.	$\frac{\text{Mg. Fe}}{\text{Mg. N}}$	Per cent hydrolysis
1287	1.55	11
1287, Fraction A	1.04	27
1287, " C	0.00	52

Effect of Trypsin on Ferritin and Apoferritin—Tryptic digestion was carried out with crystalline trypsin containing approximately 50 per cent MgSO_4 , by the method of Anson (16) with use of the Folin phenol reagent, on aliquots of the trichloroacetic acid filtrates. The protein substrates were pretreated in several different ways in order to discover the effects of these treatments on the effectiveness of trypsin. In the control, the proteins were not pretreated at all but were brought to pH 7.5 for tryptic digestion. In the remaining tubes, the protein was treated with alkali alone (pH > 12), urea alone (final concentration 40 per cent by weight), or urea plus alkali, as recommended by Anson for hemoglobin. After the preliminary treatment, the mixtures were brought to pH 7.5. Each ml. of the digestion mixture contained 1.65 mg. of protein N and 2 mg. of crystalline trypsin- MgSO_4 . Digestion was carried out at 35° for 30 minutes. The results in Table VI clearly indicate the correlation between tryptic digestion and severity of treatment required for denaturation.

Effect of pH on Vasodepressor Effect of Ferritin—The effect of pH on the denaturation (insolubility at its isoelectric point) of ferritin led to a determination of the effect of pH on the vasodepressor activity of ferritin. The lowest concentration of ferritin (crystallized with CdSO_4) which will give a clearly positive vasodepressor effect by the rat mesoappendix test is equivalent to 0.0005 γ of ferritin N per 0.5 ml. Solutions of ferritin containing 300 γ of ferritin N were incubated at 37° for 1 hour at different reactions, brought to pH 7.4, and diluted for testing to a concentration of 0.0005 γ of N per 0.5 ml. At pH 3.0 or less, the vasodepressor activity was destroyed, but from pH 3.5 to 8.0 it was strongly positive. At a pH greater than 8, the activity was destroyed. This inactivation of vaso-

TABLE VI

Effect of Trypsin on Ferritin and Apoferritin

The method of Anson (16) was used with crystalline trypsin. Incubation with NaOH alone, urea alone, or urea + NaOH for 30 minutes at room temperature. Tryptic digestion was carried out with 1.68 mg. of protein N per ml. and 4 mg. per ml. of a trypsin preparation containing 50 per cent MgSO_4 . All proteins were brought back to pH 7.5 for the enzyme action. The extent of hydrolysis is expressed in terms of per cent of the original Folin phenol color value obtained in the trichloroacetic acid filtrates after digestion for 30 minutes at 35°, corrected for the blank value.

Treatment prior to addition of trypsin	Per cent of original Folin color value		
	Ferritin	Apoferritin	Hemoglobin
None.....	20	19	12
NaOH	28	25	38
Urea	25	22	42
NaOH + urea.....	56	59	93

depressor activity appears to correspond roughly in the acid region with the extent of denaturation, but is more sensitive to alkaline treatment than is indicated by the extent of denaturation in that pH region.

Distribution of Amino Acids in Ferritin and Apoferritin—There are two references in the literature by Tria (17) and Kuhn *et al.* (18), concerning the amino acid content of ferritin. The values reported are completely at variance with each other. In light of the purity tests applied to our crystalline ferritin preparations, the reported presence in ferritin by Kuhn *et al.* of a nucleoprotein, and a denial of this latter finding by Granick and Michaelis (5), a complete analysis of ferritin was undertaken. In the present study, horse spleen ferritin recrystallized four times was reprecipitated four times at 50 per cent saturation with ammonium sulfate and freed of inorganic ions by dialysis. This solution was used for the prepara-

tion of crystalline apoferritin, which was treated in a similar manner. Both proteins were homogeneous electrophoretically. Apoferritin was used for the complete analysis rather than ferritin, to avoid difficulties from the iron, which interferes with many of the analyses. However, five amino

TABLE VII

Distribution of Amino Acid Nitrogen of Apoferritin and Ferritin

The amino acid content of these proteins was determined, for the most part, by the microbiological procedure of Henderson and Snell (11). Chemical methods were used for tyrosine, tryptophan, and cystine. The serine content was not determined. The results are expressed as the per cent of the total nitrogen accounted for by each amino acid.

Amino acid	Apoferritin*		Ferritin
	gm. per 100 gm. protein	per cent of total N	per cent of total N
NH ₃ -N.		10.0	
Humin N.....		3.4	
Glutamic.....	17.2	10.1	9.9
Aspartic.....	6.8	4.4	
Lysine.	7.8	9.2	
Arginine	9.1	18.0	
Histidine	4.8	8.0	
Cystine	1.7	1.2	
Methionine.....	1.9	1.1	1.0
Tyrosine.....	5.0	2.4 (2.2)†	
Phenylalanine.	6.1	3.2	3.0
Leucine	19.1	12.6	12.5
Isoleucine.....	1.4	0.9	
Glycine....	3.4	3.9	
Valine.....	4.3	3.2	3.1
Alanine.....	1.9	1.8	
Threonine.....	4.3	3.1	
Proline.....	1.5	1.1	
Tryptophan.....	1.2	1.0	

* The apoferritin contained, in per cent on a dry weight basis, 16.2 N, 0.0 Fe, 0.05 P, 0.16 Cd, 0.89 total S. Cystine and methionine accounted for 98 per cent of the total S present. The amino N was 5.0 per cent of the total N.

† The value of 2.2 was obtained by the chemical method (12), 2.4 by the microbiological method (11).

acids were determined in ferritin for comparison. Most of the amino acids were determined microbiologically, by use of the uniform medium of Henderson and Snell (11). Tyrosine and tryptophan (12) and cystine (13) were determined chemically. Serine was not determined. Values for the amino acid nitrogen distribution are presented in Table VII. In all, 98 per cent of the total N of apoferritin was accounted for by the usual

amino acids, free ammonia, and humin; serine was not determined. The free ammonia corresponds to that which would be liberated during hydrolysis if all of the glutamic acid existed as glutamine in the protein. The total S was accounted for to the extent of 98 per cent as total cystine plus methionine. The relative amounts of acidic and basic amino acids correspond to a protein with an acid isoelectric point. The amino acid analyses of ferritin agree with the values for apoferritin very well, and it is difficult to conceive of any nucleic acid fragments in ferritin as reported by Kuhn *et al.* The value for arginine, calculated in terms of per cent of apoferritin, as reported by Tria is 5.7 per cent, by Kuhn 16 per cent, and by Archibald (19), by the arginase method, 8.3 per cent. Our value calculated on the same basis is 9.1 per cent.

DISCUSSION

The results of the chemical studies emphasize the unique character of ferritin, a protein with over 20 per cent by weight of iron, which is present in many tissues in the dog (14), such as spleen, liver, bone marrow, kidney cortex and, to a smaller extent, in testes and skeletal and cardiac muscle. The protein is absent from normal circulating plasma or red cells. Those data which concern the effect of surface groups, such as electrophoretic mobility, viscosity, and immunochemical reactions, suggest the probability that the iron in ferritin is present in the molecule in such a manner that it does not affect the surface properties of ferritin directly. The ease with which the animal can call on this iron for purposes of iron transport and hemoglobin synthesis (1) suggests an enzymatic reaction which would release the iron from its relatively tight linkage in ferritin.

The variability of the Fe:N ratio in fractions easily obtainable from crystalline ferritin by gentle methods, such as $(\text{NH}_4)_2\text{SO}_4$ fractionation or high speed centrifugation, indicates again the aggregated state of ferritin and the fact that the iron or phosphorus can have no specific linkage with any part of the protein moiety such as some particular amino acid. This finding, together with the results of the amino acid analyses, confirms the conclusion that ferritin is not, nor does it contain, a nucleoprotein. The amino acid distribution in ferritin is quite different from that in hemoglobin, and, although the iron of ferritin may be used for hemoglobin synthesis, the protein moiety cannot be used *per se*. The vasodepressor activity of ferritin is associated with the protein moiety of ferritin, since all fractions with variable Fe:N ratios, as well as apoferritin which contains no iron, are equally active in the rat mesoappendix test. These facts do not, however, rule out the possible rôle of iron in the vasodepressor activity of ferritin, since the injection of apoferritin or a ferritin with a low

iron content may be accompanied *in vivo* by a combination with iron to form the active material.

The inability of pepsin or trypsin to digest ferritin, unless it is denatured, agrees with similar findings in the literature (20-22) concerning these two enzymes. Tria and Torboli (23) reported that ferritin was not hydrolyzed by trypsin, although after denaturation with HCl it was hydrolyzed (24). That ferritin is more difficult to denature by acid than apoferritin suggests a protective function for the iron. It should also be pointed out that during the course of its preparation ferritin solutions may be heated to 80° without denaturation. The effect of acid on the denaturation process is associated with a corresponding destruction of the vasodepressor activity of ferritin. On the other hand, treatment of ferritin at pH 9 also inactivates the vasodepressor activity of ferritin, although this treatment in the absence of urea does not denature ferritin to any extent. The alkaline inactivation appears to be concerned more with some chemically sensitive grouping in the protein molecule, or with some disturbance in the protein structure similar to denaturation, without an accompanying insolubility of ferritin at its isoelectric point.

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SUMMARY

Electrophoretic data gave isoelectric points of 4.4 for horse spleen ferritin and apoferritin, 5.2 for dog liver ferritin, and 5.4 for human liver ferritin.

Viscosity studies, together with the electrophoretic and immunochemical behavior of ferritin, indicate that the iron in ferritin is without effect on the surface-reactive groups of the protein molecule as measured by these methods.

Crystalline pepsin and trypsin will digest ferritin if the protein is first denatured. The iron in ferritin protects the protein to some extent from acid denaturation.

The amino acid distribution of ferritin precludes the presence of any nucleoprotein. All of the sulfur is accounted for by cystine plus methionine. The amino acid distribution is quite different from that of hemoglobin.

The vasodepressor activity of ferritin is dependent on the protein moiety of the molecule, but the data do not preclude the possible rôle of iron. Various fractions obtained either with ammonium sulfate or by high speed centrifugation, although they contain different amounts of iron, are crystallizable with CdSO_4 and have the same vasodepressor activities when meas-

ured by the rat mesoappendix test. The vasodepressor activity of ferritin is destroyed by a pH more acid than 3.5 or more alkaline than 8.0. In the acid region, this inactivation corresponds approximately with the extent of denaturation, but in the alkaline region the presence of some chemically sensitive grouping on the protein molecule is suggested.

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THE RELATION OF SULFHYDRYL GROUPS IN FERRITIN TO ITS VASODEPRESSOR ACTIVITY*

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Following the identification of ferritin as the hepatic vasodepressor (1) which is present in the plasma of dogs in the hyporeactive, irreversible phase of hemorrhagic shock, immunochemical (2) and chemical (3) studies were carried out with a view to determining the relation of its chemical constitution to its activity as a vasodepressor principle. The activity, as assayed by the rat mesoappendix technique of Zweifach and Chambers (4), was associated with the protein moiety of the ferritin molecule. The vasodepressor activity of ferritin was inactivated in acid solution to an extent which corresponded to the degree of denaturation. Inactivation took place more readily at a mildly alkaline pH, at which little denaturation takes place. The latter observation, together with the fact that the inactivation of the vasodepressor activity of ferritin by liver slices takes place only in oxygen, whereas its formation occurs under anaerobic conditions, led to the exploration of the possible rôle of the sulfhydryl groups in its vasodepressor activity. The results of this study support the concept that the vasodepressor activity of native ferritin is dependent on free sulfhydryl groups and that the oxidation of these sulfhydryl groups to the disulfide form is responsible for the loss of activity. This inactivation is reversible by cysteine.

EXPERIMENTAL

Crystalline ferritin was prepared from horse spleen as previously described (1). Assays for vasodepressor activity were carried out by the rat mesoappendix technique (4). Rabbit antiserum to crystalline dog ferritin was prepared and used by methods already described (2). *o*-Iodosobenzoic acid was prepared by the method of Meyer and Wachter (5) as modified by Loevenhart and Grove (6), recrystallized several times, and assayed by the iodine titration method of Hellerman *et al.* (7).

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Iodoacetamide was prepared by the method of Anson (8). *p*-Chloromercuribenzoate was prepared according to Whitmore and Woodward (9). Reduced glutathione was isolated from yeast according to the methods of Hopkins (10) and of Kendall *et al.* (11). Its purity was assayed by iodine titration. Crystalline reduced glutathione was obtained from the Eastman Kodak Company for purposes of comparison. Oxidized glutathione was

TABLE I

Inactivation and Reactivation of Ferritin

The mixtures for inactivation contained 0.15 mg. of ferritin N per ml. and were 0.0004 M with respect to the sulfhydryl-reacting agent in 0.1 M phosphate buffer, pH 7.0. Incubation was at 37.5° for 30 minutes. For reactivation the above mixtures, after preliminary incubation, were treated with an equal volume of a solution, containing 0.3 mM per ml. of cysteine and incubated further for 30 minutes. For testing, each solution was diluted so as to contain 0.001 γ of ferritin N per 0.5 ml. or an equivalent amount of the reagent and injected into the test rat.

Incubation mixture	Vasodepressor activity
Ferritin	+
" + <i>o</i> -iodosobenzoate	-
" + O ₂ + Cu ⁺⁺	-
" + iodoacetamide	-
" + <i>p</i> -chloromercuribenzoate	-
" + ferricyanide	+
Ferritin + <i>o</i> -iodosobenzoate + cysteine	+
" + O ₂ + Cu ⁺⁺ + cysteine	+
" + iodoacetamide + cysteine	+
" + <i>p</i> -chloromercuribenzoate + cysteine	+
<i>o</i> -Iodosobenzoate	-
Iodoacetamide	-
<i>p</i> -Chloromercuribenzoate	-
Cu ⁺⁺	-
Cysteine	-
Ferricyanide	-

obtained by oxidation of reduced glutathione in mildly alkaline solution by a current of oxygen.

Effect of Sulfhydryl-Reacting Compounds on Vasodepressor Activity of Ferritin—The effects of various agents which are known to react with protein sulfhydryl groups were tested with respect to their ability to abolish the vasodepressor activity of crystalline ferritin as measured by the rat mesoappendix test. A mixture was prepared which contained 0.15 mg. of ferritin nitrogen per ml., together with the sulfhydryl-reacting agent, the

latter in a final concentration of 0.0004 M in 0.1 phosphate buffer, pH 7.0. Prior to injection into the test rat, this mixture was incubated at 37.5° for 30 minutes and diluted with saline for testing so as to contain 0.001 γ of ferritin nitrogen per 0.5 ml., which was the amount injected. Control tests were carried out with untreated ferritin as well as with solutions of the sulfhydryl-reacting agents alone. The results are shown in Table I. *o*-Iodosobenzoate as well as oxygen in the presence of cupric ion served to inactivate the vasodepressor effect of ferritin. Oxygen alone was ineffective. The effect of these reagents consists in an oxidation of protein sulfhydryl groups to the disulfide form. Inactivation was also obtained with iodoacetamide and *p*-chloromercuribenzoate, which chemically bind the sulfhydryl groups. Ferricyanide did not affect the activity of ferritin.

In the reactivation experiments, the solution of ferritin and sulfhydryl-reacting reagent was first incubated for 30 minutes. It was then mixed with an equal volume of a freshly prepared and neutralized solution of cysteine hydrochloride containing 0.3 mM per ml. and incubated for an additional 30 minutes. The solution was then appropriately diluted and tested for vasodepressor activity. Cysteine proved capable of reversing the inactivation of ferritin brought about by the sulfhydryl-reacting reagents. Control assays on rats were made of all reagents at equivalent concentrations and were found to be negative for vasodepressor activity.

It was of interest that the inhibition of ferritin activity by iodoacetamide was reversible by cysteine. It was thought possible that this might result not from a reversal of the iodoacetamide reaction but from a reduction by cysteine of some disulfide bonds present in the original ferritin. This possibility was excluded by repeating with apoferritin the inactivation with iodoacetamide and the reactivation with cysteine. Since apoferritin is prepared from ferritin by treatment with sodium hydrosulfite, any disulfide groups would be reduced to the sulfhydryl form. Iodoacetamide completely abolished the vasodepressor activity of apoferritin. Subsequent treatment with cysteine restored its activity to that of the original apoferritin. Hence, it may be concluded that the iodoacetamide reaction was reversed by cysteine.

Inactivation of Vasodepressor Activity of Ferritin by Cystine and Oxidized Glutathione and Reactivation by Cysteine—Ferritin was treated with cystine or with oxidized glutathione in a manner similar to that described in Table I. The cystine solution was obtained by dissolving 60 mg. of cystine in 100 ml. of hot 0.05 M sodium phosphate buffer, pH 7.4 (12). The cystine remained in solution after reaching room temperature. The original ferritin was active at a dilution equivalent to 0.0005 γ of ferritin

nitrogen per 0.5 ml. After incubation with cystine or oxidized glutathione (0.001 M final concentration) the ferritin lost its activity. The ferritin-cystine or ferritin-oxidized glutathione mixture was dialyzed against several changes of large volumes of 0.05 M phosphate buffer, pH 7.4, overnight in the ice box to remove the cystine or glutathione. The ferritin solution remained inactive. Aliquots were then incubated with cysteine, with complete recovery of the ferritin vasodepressor activity.

Sulfhydryl Content of Ferritin—An attempt was made to arrive at a quantitative estimation of the sulfhydryl groups of native ferritin in order to determine the effect of a variety of procedures on these groups. It was found by chemical measurements that native ferritin did not react measurably with ferricyanide (13), a finding which is in agreement with our earlier observation that ferricyanide does not affect the vasodepressor activity of ferritin. However, ferritin did react with *o*-iodosobenzoate (14), iodoacetamide (15), and iodine in potassium iodide (16) as evidenced by chemical measurements. The reaction with *o*-iodosobenzoate could not be carried out quantitatively, because of the intense color of the ferritin which obscured the iodine titration. Since the reaction of ferritin with iodoacetamide can be measured quantitatively after precipitation of the protein, this reaction was adopted for subsequent determinations. It was found that none of the amino acids except cysteine reacts with iodoacetamide at pH 7.4. It was possible to demonstrate in several ways that the groups in ferritin which react with iodoacetamide are sulfhydryl. A preparation of apoferritin, which was almost colorless and free from iron, gave values with *o*-iodosobenzoate for the native protein which were only 10 per cent higher than those obtained by the iodoacetamide method. Furthermore, the treatment of ferritin or apoferritin with iodoacetamide decreased considerably the groups which were then capable of reacting with iodine-potassium iodide. The iodine-potassium iodide undoubtedly reacted with groupings other than sulfhydryl, since the total would have been much greater than the total cysteine content of ferritin, on the assumption that all of the cystine sulfur was in the reduced form. Finally, pretreatment of ferritin or apoferritin with an excess of the very specific sulfhydryl reagent, *p*-chloromercuribenzoate, abolished the subsequent reaction of the proteins with iodoacetamide. The protein was incubated at pH 7.4 with increasing quantities of *p*-chloromercuribenzoate for 10 minutes and then treated with iodoacetamide. As the quantity of *p*-chloromercuribenzoate was increased, the subsequent reaction of the treated ferritin with iodoacetamide decreased until a point was reached at which the reaction with iodoacetamide was completely abolished.

The quantitative method of Rosner (15) with iodoacetate was modified for the estimation of ferritin sulfhydryl groups by the substitution of

iodoacetamide. The hydrogen iodide formed was converted to free iodine and determined colorimetrically with a Klett-Summerson photo-colorimeter and a No. 42 filter. Time curves were run on a mixture of 5 ml. of a ferritin solution in phosphate buffer, pH 7.0 or 7.4, together with 5 ml. of a 0.2 M neutralized solution of iodoacetamide. At the appropriate times, 0.25 ml. of a 100 per cent trichloroacetic acid solution was added with vigorous shaking. The precipitated ferritin was filtered and the entire water-clear filtrate treated with 0.1 ml. of concentrated sulfuric acid. An aliquot of 5 ml. was treated with 0.1 ml. of a fresh 3 per cent hydrogen peroxide solution and the yellow iodine color read after 20 minutes at room temperature. Results similar to those of Rosner were obtained (Fig. 1), supporting the inference that after 10 minutes all of the sulfhydryl groups had reacted. After this time, more iodoacetamide reacted with unknown groupings in the protein molecule (17) but at a much reduced

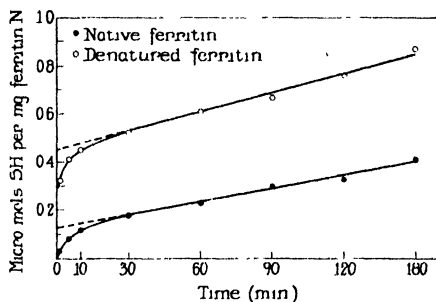


Fig. 1. Reaction of ferritin with iodoacetamide, pH 7.0, at room temperature. Denatured ferritin prepared by treatment of native ferritin with urea and alkali.

and more uniform rate, as indicated by the almost linear relationship with time. In all subsequent experiments, the ferritin solution was allowed to react with iodoacetamide for 10 minutes and the hydrogen iodide liberated taken as the index of the sulfhydryl groups in the protein. A number of samples of ferritin and apoferritin were tested by this technique and were all found to contain sulfhydryl groups. Among these were crystalline ferritins of horse spleen, horse liver, dog liver, and human liver and apoferritins of horse spleen and dog liver.

A solution of native horse spleen ferritin contained $27 \mu\text{M}$ of sulfhydryl per 100 mg. of ferritin nitrogen as measured by iodoacetamide at pH 7.4. On denaturation with urea and alkali (3), the sulfhydryl content was increased to a value of $55 \mu\text{M}$. The solution of native ferritin was then incubated with excess reduced glutathione for 1 hour at room temperature and dialyzed exhaustively to remove the excess glutathione. The sulfhydryl content of the ferritin solution was $35 \mu\text{M}$ per 100 mg. of ferritin nitro-

gen. Denaturation of this ferritin solution resulted in an increase of sulfhydryl to a value of 64. Since the reaction of iodoacetamide and the sulfhydryl groups of native ferritin is sufficient to inactivate the vasodepressor activity of ferritin, one can conclude that, of the sulfhydryl groups in the ferritin molecule, those available for reaction with iodoacetamide when ferritin is in the native state include those concerned with its vasodepressor activity.

Effect of Cystine on Sulfhydryl Content of Ferritin—In order to show that the action of cystine which inactivates the vasodepressor activity of ferritin is associated with a reduction of ferritin sulfhydryl groups, 60 ml. of ferritin, containing 8.0 mg. of nitrogen per ml., were dialyzed against 1 liter of a solution of 600 mg. of cystine in 0.05 M sodium phosphate buffer, pH 7.4 (12), overnight at room temperature. The ferritin was then dialyzed against many changes of large volumes of water to remove excess cystine. The sulfhydryl content of the ferritin before treatment with cystine was $31.7 \mu\text{M}$ per 100 mg. of ferritin nitrogen. After treatment with cystine, it had fallen to $20.3 \mu\text{M}$ of sulfhydryl per 100 mg. of ferritin nitrogen.

Sulfhydryl Content and Vasodepressor Activity of Ferritin Treated with Reduced Glutathione—The sulfhydryl groups available for reaction with iodoacetamide at pH 7.4 are increased after treatment with glutathione. This effect can be interpreted as due either to the reduction of disulfide linkages in the native protein or to the liberation of sulfhydryl linkages previously blocked by cadmium, which is used as CdSO_4 for the crystallization procedure and which cannot be removed completely by dialysis or reprecipitation with ammonium sulfate. Previous studies with crystalline ferritin (1) have shown that such ferritin solutions were active in the mesoappendix test when injected into the test rat in a concentration of 0.0001 to 0.0005 γ of ferritin nitrogen per 0.5 ml. The ferritin which had been treated with glutathione and dialyzed was now active at a concentration of 0.00005 γ of ferritin nitrogen per 0.5 ml., an increase in activity of 2 to 10 times.

Vasodepressor Activity of Glutathione and Iron Salts—Early in this study, it was observed that reduced glutathione *per se* gave a positive vasodepressor effect in the test rat at a concentration of 0.005 γ per 0.5 ml. This concentration is approximately equivalent, in terms of nitrogen, to the ferritin concentration required to give a positive vasodepressor test. Oxidized glutathione was inactive over a wide range of concentrations. In view of these observations, in all of the experiments with ferritin solutions treated with reduced glutathione, the final concentration of glutathione in the reaction mixture was reduced by dialysis or other means to levels well below its range of activity as a vasodepressor. A second

observation of interest was that inorganic iron also proved to be an active vasodepressor. Colloidal ferric oxide, ferric chloride, or ferrous sulfate gave positive vasodepressor tests at concentrations of 0.001 to 0.005 γ of iron per 0.5 ml.

The possibility was explored that the vasodepressor effects of both reduced glutathione and iron might in some manner be mediated via endogenous ferritin. Thus the injection of iron might lead to its combination with apoferritin to form ferritin, and the reduced glutathione might produce its vasodepressor effect through a reduction of inactive ferritin. If these explanations were true, the vasodepressor effects obtained with both iron and glutathione would be inhibited in the presence of an antibody to ferritin. Although it would have been preferable to test this hypothesis by the use of antiserum to rat ferritin, no antiserum was available. Fortunately, extracts of rat liver containing ferritin yield positive precipitin tests with rabbit antiserum to either dog or horse ferritins. A concentrated solution of a globulin fraction of rabbit antiserum to horse ferritin was used. The method of testing was that employed in a previous report (2). It consisted of the injection into a test rat of 0.5 ml. of the antibody solution 30 minutes prior to the injection of 0.5 ml. of either iron or reduced glutathione. In both cases, the vasodepressor responses were either abolished completely or markedly reduced. These findings support the inference suggested above as to the manner in which iron or glutathione exerts its vasodepressor effect.

Inactivation of Ferritin by Specific Antiserum—The possibility was explored that the inactivation of the vasodepressor activity of ferritin (2) by specific rabbit antiferritin serum involves a reaction of ferritin with its antibody in such a manner that its sulfhydryl groups are blocked. If this were true, treatment of this inactivated ferritin with excess cysteine might bring about a restoration of the vasodepressor activity. This was not the case. A solution of ferritin containing 0.0005 γ of nitrogen gave a strong positive vasodepressor response in the test rat. Treatment of such ferritin with a diluted antiferritin serum brought about its complete inactivation when tested at the same concentration of ferritin nitrogen. With this concentration of ferritin, no observable precipitate was produced on addition of antiserum. The antiserum-inactivated ferritin solution was then treated with cysteine, but no reactivation was obtained. Subsequent experiments have shown that ferritin, which was oxidized so that its sulfhydryl content was reduced without denaturation, reacted quantitatively to the same degree with antiferritin serum as did the untreated ferritin.

Effect of Cysteine and Reduced Glutathione on Ferritin Iron—The only method hitherto reported for the removal of ferritin iron without denatura-

tion of the protein is repeated treatment at pH 4.6 with sodium hydro-sulfite (18). Under these conditions, the iron is reduced to the ferrous state, as evidenced by a positive color reaction given by the dialysate with α, α' -dipyridyl when such a mixture is dialyzed. During our experiments with cysteine-treated ferritin at pH 7.4, it was noted that a copious precipitate of cystine formed rapidly, out of proportion to the amount of cysteine which could be oxidized by the protein disulfide groups or by the oxygen in the air. The color of the deproteinized solution was reminiscent of the reaction of ferrous iron with cysteine reported by Michaelis (19), which leads to the autocatalytic oxidation of all of the cysteine to cystine. On further study, it was found that cysteine and reduced glutathione at pH 7.4 were capable of bringing about the reduction of ferritin

TABLE II

Effect of Cysteine and Glutathione on Ferritin Iron

3 ml. of a ferritin solution containing 7.40 mg. of total iron were treated with 3 ml. of water or of each of the reducing agents, all at pH 7.4, at 37.5° for 2 hours. An equal volume of a saturated ammonium sulfate solution was added to each and the mixture centrifuged at 4700 r.p.m. Aliquots of each supernatant were analyzed for ferrous iron by the α, α' -dipyridyl method (20). The values reported are corrected for the amount of ferrous iron in the control ferritin-water mixture.

Concentration of reducing agent <i>mm per 6 ml.</i>	Ferrous iron formed by	
	Cysteine <i>mg. per 6 ml.</i>	Glutathione <i>mg. per 6 ml.</i>
1.5	3.899	0.199
0.15	0.673	0.013
0.015	0.046	0.010

iron and its removal from its relatively tight combination with the protein. 3 ml. of a ferritin solution were mixed with 3 ml. of water or of each of the reducing agents after adjustment to pH 7.4 and incubated for 2 hours at 37.5°. The mixtures were treated with 6 ml. of a saturated solution of ammonium sulfate and centrifuged at 4700 r.p.m. for 5 minutes. Aliquots of the supernatant solution were treated with α, α' -dipyridyl for the determination of ferrous iron (20). The ferritin-water control yielded traces of color with the reagent and this was subtracted from each of the experimental values. The results are given in Table II. Cysteine was more effective than glutathione at equivalent concentrations.

DISCUSSION

Evidence for the conclusion that the vasodepressor activity of ferritin is related to its sulfhydryl groups is dependent on the specificity of the

reagents used to react with the sulfhydryl groups in ferritin. The reagents, which were selected for this study and found to inactivate ferritin, were those which have been used conventionally to combine with or oxidize the sulfhydryl groups of proteins. It may be pointed out that, although these reagents react most rapidly with the sulfhydryl group of proteins and of amino acids containing the sulfhydryl group, several also react with other groupings in the protein molecule, although at a slower rate. However, reagents such as cystine or oxidized glutathione are without this disadvantage, inasmuch as they react only with the sulfhydryl group in proteins. The concept of the specificity of sulfhydryl groups in relation to the vaso-depressor activity of ferritin is further enhanced by the reactivation of inactive ferritin by cysteine and the augmentation of ferritin activity by reduced glutathione.

With respect to iodoacetic acid, and to a lesser extent iodoacetamide, it has been reported (21) that these are less satisfactory reagents for the detection of sulfhydryl groups in certain proteins. For example, some protein enzymes which are strongly inhibited by *p*-chloromercuribenzoate, *o*-iodosobenzoate, and organic arsenicals are only slightly inhibited by iodoacetamide, indicating that certain sulfhydryl groups in these proteins are incapable of reacting with iodoacetamide. In the case of ferritin, however, inactivation does occur with iodoacetamide as well as with *p*-chloromercuribenzoate and *o*-iodosobenzoate. In certain other respects, the sulfhydryl groups of ferritin resemble those of native egg albumin (22). Thus, native ferritin does not react with ferricyanide, although it reacts with iodine in potassium iodide. The fact that iodoacetamide reacted quantitatively with sulfhydryl groups in the same way as *p*-chloromercuribenzoate permitted the use of iodoacetamide for the determination of sulfhydryl groups in the highly colored ferritin solutions. The ease with which ferritin sulfhydryl groups may be oxidized and reduced reversibly suggests that these sulfhydryl groups are present in the protein molecule in such a pattern as to partake in these reactions without an irreversible alteration in the structure of the protein.

Standard preparations of crystalline ferritin differ in their sulfhydryl content. This variability can be attributed to the different periods of anaerobiosis to which the tissues were subjected before extraction of ferritin, since evidence is available that the sulfhydryl form of ferritin progressively increases in liver during anaerobic incubation. An additional factor may be the cadmium still attached to ferritin even after reprecipitation with ammonium sulfate and exhaustive dialysis. The sulfhydryl content of such a ferritin preparation can be further increased by treatment with reduced glutathione, indicating a liberation of additional sulfhydryl groups from ferritin-disulfide linkages or from combination with cadmium.

It is relevant that the highest content of sulfhydryl groups in the native protein is obtained with apoferritin after reduction of ferritin with sodium hydrosulfite and subsequent precipitation at 50 per cent saturation with ammonium sulfate without the use of cadmium sulfate. As with many other proteins (8, 23, 24), denaturation of ferritin or apoferritin makes the greatest number of sulfhydryl groups available for reaction with iodoacetamide. The nitroprusside reaction cannot be performed on ferritin because of its intense color. In the case of apoferritin, this color reaction is weak or negative until denaturation occurs, whereupon it becomes strongly positive.

Data in this and previous studies indicate that the protein moiety of ferritin has an essential rôle in its vasodepressor activity. Thus, apoferritin has equivalent vasodepressor activity to ferritin and the effects of both can be abolished by specific antiferritin serum. However, since the biological effects of ferritin and apoferritin are measured in the intact animal, the possibility is not precluded that both may function as vasodepressors by virtue of their capacity to carry iron to their site of action, the smooth muscles of the peripheral blood vessels. This possibility is supported by the finding that the intravenous administration of ferrous or ferric iron in amounts comparable to those present in ferritin produces equivalent vasodepressor effects.¹ That these effects could come about through union of iron with apoferritin is suggested by the prevention of the vasodepressor activity of iron by the prior injection of antiferritin serum. However, the inactivity of disulfide ferritin would indicate that the mere presence of iron in the molecule is not sufficient to bring about a vasodepressor effect. It would therefore be necessary for this ferritin molecule to be altered in such a manner that the iron-ferritin linkage would be made labile. It may be relevant to this hypothesis that treatment of ferritin with reduced glutathione or cysteine, which activates the protein, also labilizes the ferritin iron, converting it to the ferrous state. Also pertinent is the observation that reduced glutathione *per se* has a vasodepressor effect in the intact animal which can be prevented by prior injection of the test rat with antiferritin serum.

SUMMARY

The vasodepressor activity of ferritin is associated with the presence of sulfhydryl groups in the native protein. The reaction of ferritin with reagents known to combine with or oxidize the sulfhydryl group of proteins

¹ During the preparation of this paper Crismon reported (25) that the intravenous injection of 91 γ per kilo of ferrous iron raised the epinephrine threshold of the capillary bed in the exteriorized rat mesoappendix 3- to 15-fold, thus inducing a marked vasodepressor response. He likewise suggested the combination of iron with apoferritin to produce this effect.

leads to its inactivation. Among these reagents are *o*-iodosobenzoate, oxygen with a trace of Cu^{++} , cystine, oxidized glutathione, *p*-chloromercuribenzoate, and iodoacetamide. After inactivation, ferritin may be reactivated by treatment with excess cysteine.

The sulfhydryl content of ferritin or apoferritin is determined by reaction with iodoacetamide. An increase in sulfhydryl content of ferritin is obtained after treatment with reduced glutathione, followed by dialysis, whereas the sulfhydryl content of ferritin is decreased after treatment with cystine followed by dialysis. Apoferritin, prepared from ferritin by reduction with sodium hydrosulfite, has the highest sulfhydryl content for the native protein. Denaturation of ferritin increases the number of sulfhydryl groups which react with iodoacetamide.

Treatment of ferritin with cysteine or reduced glutathione at physiological pH (7.4) not only reduces the disulfide groups to the sulfhydryl state but also brings about a reduction of ferritin iron to the ferrous state which makes the ferrous-ferritin linkage more labile.

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OXIDATION AND REDUCTION OF FERRITIN SULFHYDRYL-DISULFIDE GROUPS BY LIVER*

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Previous studies in our laboratory (1) have shown that the vasodepressor activity present in the plasma of dogs in the hyporeactive, irreversible phase of hemorrhagic shock was due to a substance formed by the liver as a result of tissue hypoxia. A substance with similar vasodepressor activity could also be obtained *in vitro* by incubation of normal liver slices in a Ringer-phosphate solution under anaerobic conditions, whereas under aerobic conditions normal liver slices were capable of inactivating this vasodepressor material (VDM). These findings are summarized in the following reactions.

VDM Formation—	(A)	Normal liver slices	+	O ₂	→	no VDM
	(B)	“ “ “	+	N ₂	→	VDM
	(C)	“ “ “	+	VDM + O ₂	→	no VDM
	(D)	“ “ “	+	“ + N ₂	→	VDM

The vasodepressor material of hepatic origin was identified as ferritin (2) and its activity was found to be associated with the presence of free sulfhydryl groups (3). Oxidation or blocking of the ferritin sulfhydryl groups by various reagents resulted in the inactivation of the vasodepressor effect of ferritin. The inactivated ferritin could be reactivated with excess cysteine. The present experiments with surviving liver slices show that the activation and inactivation of ferritin are also accompanied by reduction and oxidation, respectively, of its disulfide and sulfhydryl groups.

These findings are suggestive of an equilibrium in the intact hepatic cell between the active and inactive forms of ferritin, an equilibrium which can be altered in response to various circulatory states and changes in oxygen tension.

EXPERIMENTAL

Chemical State of Sulfhydryl-Disulfide Groups of Ferritin in Normal Dog Liver Aerobically and Anaerobically—Previous reports from this laboratory

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have shown that the vasodepressor activity of ferritin is found in the medium when normal liver slices are incubated in nitrogen but not in oxygen (see Reactions A and B). In relation to the hypothesis that the vasodepressor activity of ferritin is determined by the state of sulfhydryl-disulfide groups, it was of interest to determine the state of these groups in the normal cell under aerobic as well as anaerobic conditions. With this in mind, a normal dog was anesthetized with pentobarbital and a portion of its liver removed and frozen immediately in carbon dioxide snow. Three other portions of the same liver lobe were placed in flasks and incubated at 37.5° in a moist atmosphere of nitrogen for 30, 60, and 120 minutes, respectively. At the appropriate times, each liver specimen was frozen. All four samples were treated similarly from this point on, in order to obtain a solution of partially purified ferritin. 20 gm. of the frozen liver sample were powdered in a stainless steel mortar which was surrounded with carbon dioxide snow. The frozen powder was transferred to 50 ml. of 0.1 M acetate buffer, pH 5.0, and heated immediately with stirring in a boiling water bath to 75°. The suspension was filtered through cheese-cloth, cooled in an ice bath, and centrifuged at 4700 r.p.m. in a cold centrifuge. The clear supernatant was treated with an equal volume of saturated ammonium sulfate and centrifuged again. The supernatant was discarded and the precipitate dissolved in 10 ml. of water. The ammonium sulfate precipitation was carried out four times in all. The final precipitates were each dissolved in 10 ml. of water and dialyzed against distilled water until free of ammonium sulfate. The concentration of ferritin in each solution was determined immunochemically. Aliquots of each solution were mixed with excess rabbit anti-dog ferritin serum and the precipitated ferritin-antibody complex treated in a manner previously described (4). The total nitrogen in the washed specific precipitates was compared with a standard curve constructed from values obtained with the same antiserum and known amounts of crystalline dog liver ferritin. The ferritin nitrogen content of liver Fraction A (frozen immediately) was 0.025 mg. per ml., of Fraction B (30 minutes in nitrogen) 0.027 mg. per ml., of Fraction C (60 minutes in nitrogen) 0.021 mg. per ml., and of Fraction D (120 minutes in nitrogen) 0.022 mg. per ml. Hence, there was no significant difference in the total ferritin concentrations in the various samples.

Table I gives the results of the tests for vasodepressor activity (5) on diluted samples of each fraction. Enough tests were carried out on serial dilutions of these solutions to determine the lowest concentration of total ferritin nitrogen capable of giving a positive vasodepressor test of standard intensity. It may be seen that, per unit of ferritin in solution, Fraction A was the least active. Those fractions maintained for varying periods

of time in nitrogen became progressively more active, maximal activity being attained at 60 minutes of anaerobic incubation. The activity of Fractions C and D, corresponding to a concentration of 5×10^{-5} γ of ferritin nitrogen per 0.5 ml., agrees with the activity found for crystalline ferritin after treatment with glutathione. All fractions were then incubated at 37.5° for 30 minutes with cysteine in the proportion of 1 ml. of ferritin solution to 0.1 ml. of neutralized cysteine hydrochloride solution containing 5 mg. of the hydrochloride. These were then diluted for testing and the activities determined as before. The solutions, after cysteine treatment, were all active at a concentration of 5×10^{-5} γ of ferritin nitrogen per 0.5 ml. These results indicate that ferritin is present in the normal liver cell almost exclusively (99 per cent) in the disulfide form, which is inactive as a vasodepressor, but that under anaerobic conditions it is progressively converted to the sulfhydryl or active form.

TABLE I

Comparative Vasodepressor Activities of Ferritin from Aerobic Versus Anaerobic Dog Liver

Fraction	Vasodepressor activity		Sulfhydryl ferritin in liver
	Original solution	After cysteine treatment	
	γ ferritin $N \times 10^{-5}$	γ ferritin $N \times 10^{-5}$	per cent of total ferritin
A. Aerobic.....	500	5	1
B. 30 min. N_2	20	5	25
C. 60 " ".....	5	5	100
D. 120 min. N_2	5	5	100

Effect of Anaerobiosis on State of Glutathione in Dog Liver—In order to determine whether any correlation exists between sulfhydryl-ferritin and reduced glutathione in dog liver after incubation in nitrogen, a series of experiments was carried out of a similar nature to those described in the preceding section, and the concentration of GSH determined. Most chemical methods for GSH determination in tissues or body fluids are non-specific and are based on the reaction with iodine of the non-protein filtrate obtained after treatment of the tissue with a suitable protein-precipitating reagent. An example of such a method is that of Woodward and Fry (6). The method of Binet and Weller (7) has the advantage of first precipitating the GSH as a Cd complex prior to titration with iodine. Both procedures were employed in this study with similar results. Three dog livers were analyzed by the method of Binet and Weller and one by that of Woodward and Fry. The average values for GSH in terms of mg. per 100 gm. of wet weight liver were as follows: "aerobic" 138, 30

minutes in nitrogen 161, 60 minutes in nitrogen 211, and 120 minutes in nitrogen 201. These results indicate an increase in the GSH concentration of the tissue during anaerobic incubation in association with the increase in concentration of sulfhydryl ferritin.

Inactivation and Oxidation of Sulfhydryl Groups of Ferritin by Normal Liver Slices—The vasodepressor activity of ferritin is inactivated by incubation with normal rabbit, dog, or rat liver slices in 100 per cent oxygen (Reaction C). A series of experiments was carried out to determine whether this biological inactivation was also due to the conversion of ferritin sulfhydryl to disulfide groups. Table II lists the results obtained when the solution containing ferritin inactivated by liver slices in oxygen (Solution A) was treated with cysteine. Reactivation occurred, indicating that aerobic inactivation by the liver slice was due to an oxidation of

TABLE II
Inactivation of Ferritin by Liver Slices

Ferritin was diluted with Ringer-phosphate solution, pH 7.4, so as to contain 0.001 γ of ferritin N per 0.5 ml. and incubated for 2 hours at 37.5° as indicated. The solution was clarified by centrifugation at 4700 r.p.m. in a cold centrifuge before testing or incubation at 37.5° for 30 minutes with cysteine for attempted reactivation.

Incubation mixture	Vasodepressor activity
Ferritin-Ringer-PO ₄ + O ₂	+
“ + liver slices + O ₂ (Solution A).....	—
Ringer-PO ₄ + liver slices + O ₂ (Solution B).	—
Cysteine + (Solution A)	+
“ + (“ B)	—
“	—

sulfhydryl ferritin to the disulfide form. However, when the Ringer-phosphate medium in which liver slices had been incubated in oxygen (Solution B) was treated with cysteine, no vasodepressor activity was noted. This demonstrates that no ferritin, not even the inactive disulfide form, is liberated into the medium in 100 per cent oxygen.

In order to provide additional evidence that liver slices oxidize the sulfhydryl groups of ferritin under aerobic conditions, experiments were carried out on rat liver slices with relatively large quantities of ferritin as a substrate. The concentration of ferritin used approximates the protein content of serum and was found to be without effect on the respiration of normal liver slices. The ferritin solutions were first dialyzed against Ringer-phosphate solution, pH 7.4, and then diluted with Ringer-phosphate so as to contain 5 to 6 mg. of ferritin N per ml. A series of 125 ml. Erlenmeyer flasks was prepared containing 7.5 ml. of this ferritin solution

and 1.5 gm. of liver slices. The flasks were then equilibrated with 100 per cent oxygen and incubated with shaking at 37.5°. At appropriate intervals, the contents of each flask were centrifuged at 4700 r.p.m. in a cold centrifuge and a 5 ml. aliquot treated with 5 ml. of a 0.2 M iodoacetamide solution previously adjusted to pH 7.4. The estimation of sulfhydryl content was performed as described previously (3). Additional experiments were carried out with decreasing concentrations of oxygen in the gas phase. Control ferritin solutions were also incubated in the absence of liver and found to undergo no alteration in sulfhydryl content. In another control study, the sulfhydryl content of the medium obtained

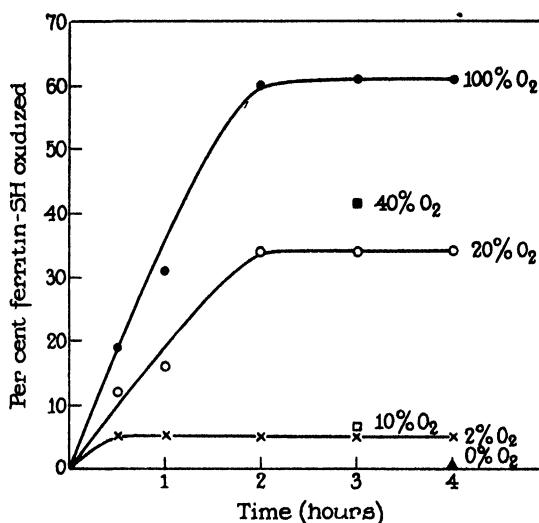


FIG. 1. Oxidation of ferritin sulfhydryl groups by normal rat liver slices at various oxygen concentrations; pH 7.4 in Ringer-phosphate.

when liver slices were incubated without the addition of ferritin was low and constant for the various time intervals studied.

The results for normal rat liver slices are shown in Fig. 1. Similar results have been obtained with dog and rabbit liver. A progressive disappearance of sulfhydryl groups was noted up to 2 hours, with no further oxidation after this time. Further treatment of this ferritin solution with fresh liver slices did not affect its sulfhydryl content. A correlation was evident between the extent of oxidation of ferritin sulfhydryl groups and the oxygen concentration in the gas phase. At zero oxygen tension (100 per cent nitrogen), no oxidation of ferritin sulfhydryl occurred; in fact, as will be shown in a later section, there is an increase in the quantity of ferritin sulfhydryl. In a similar experiment in which apoferritin instead

of ferritin was incubated with liver slices in 100 per cent oxygen, a similar decrease in sulfhydryl groups was obtained, indicating that the iron of ferritin was not concerned in this reaction.

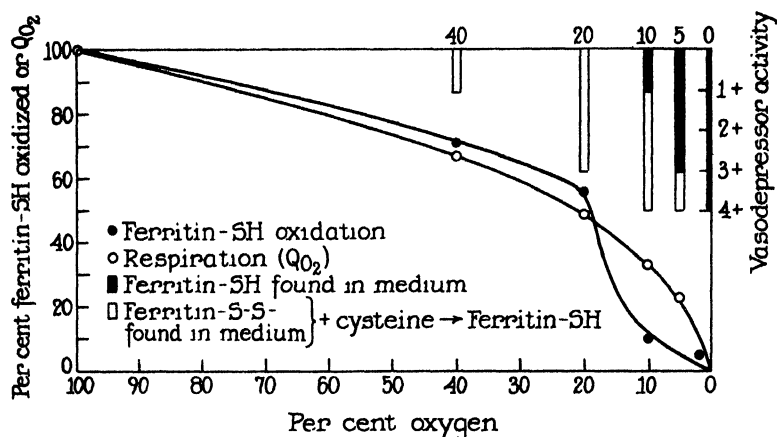


FIG. 2. Relationship of ferritin sulfhydryl oxidation, Q_{O_2} , of normal rat liver slices, and formation of active and inactive ferritin at various oxygen concentrations.

TABLE III

Relation of Ferritin Sulfhydryl Oxidation to Weight of Rat Liver Slices

7.5 ml. of a ferritin solution, previously dialyzed against Ringer-phosphate, pH 7.4, containing 5 mg. of nitrogen, were added to varying weights of surviving rat liver slices, equilibrated with 100 per cent oxygen, and incubated for 2 hours at 37.5°. A control flask containing only ferritin was incubated simultaneously. Aliquots of the ferritin solutions were then treated with iodoacetamide for estimation of their sulfhydryl content.

Liver slices	Ferritin SH oxidation
gm. wet weight	per cent of original
0.10	11
0.25	21
0.50	25
1.50	61
3.00	59

The two curves of Fig. 2 provide a comparison between the extent of ferritin sulfhydryl oxidation and the oxygen consumption of liver slices as affected by decreasing oxygen tensions. There is a correlation between the extent of oxidation of ferritin and the level of tissue respiration.

Table III shows the effect of varying the quantity of liver on the extent of ferritin sulfhydryl oxidation. There was an increase of oxidation of

the ferritin up to 1.5 gm. of liver slices per 7.5 ml. of medium. Beyond this no further increase in oxidation was observed. In all of the experiments reported this ratio of ferritin to liver slices was used.

Liver is the only tissue which has been found capable of inactivating the vasodepressor effect of ferritin (1). Liver likewise proved most active with respect to its ability to oxidize the sulfhydryl groups of ferritin. Comparative studies of several tissues from a normal dog yielded the following results, which are expressed in terms of per cent oxidation of ferritin sulfhydryl groups: liver 49, cardiac muscle 18, skeletal muscle 16, intestinal smooth muscle 16, spleen 11, and kidney cortex 7. These values were obtained by incubating 1.5 gm. of tissue slices or muscle strips in 100 per cent oxygen at 37.5° in 7.5 ml. of ferritin solution, previously dialyzed against Ringer-phosphate, pH 7.4. The ability of the various muscular tissues, spleen, and kidney cortex, to oxidize a portion of the sulfhydryl groups of ferritin without affecting its vasodepressor properties suggests that only a portion of the total sulfhydryl groups in ferritin which react with iodoacetamide is concerned with its activity.

Effect of Anaerobic Pretreatment of Normal Liver on Its Subsequent Capacity to Oxidize Ferritin Sulfhydryl Groups—Reaction D shows that normal liver slices in nitrogen are incapable of inactivating the vasodepressor activity of ferritin as measured by the rat test. It had also been shown (8) that, when normal liver slices were preincubated in nitrogen and then returned to oxygen, the capacity to inactivate the vasodepressor activity of ferritin was diminished in direct proportion to the duration of the previous anaerobic exposure. The latter technique was applied to the measurement of the capacity of rat liver slices, preincubated in nitrogen for different periods, to oxidize ferritin sulfhydryl groups when returned to oxygen for 3 hours. Rat liver slices were incubated in nitrogen in 5 volumes of Ringer-phosphate, pH 7.4, at 37.5° for different periods of time. The tissue (1.5 gm., wet weight) was removed from the Ringer-phosphate solution and transferred to a flask containing 7.5 ml. of ferritin-Ringer-phosphate, pH 7.4, and reincubated for 3 hours in oxygen. The sulfhydryl content of the ferritin was determined as outlined in a previous section. In control observations, rat liver slices were preincubated for similar periods in oxygen. Livers from ten normal rats, maintained in oxygen throughout, oxidized from 52 to 61 per cent of the sulfhydryl groups of ferritin in 3 hours, with an average value of 58 per cent. After 15 minutes exposure to nitrogen this value had dropped to 53; after 1 hour, to 37; and after 2 hours it had fallen to 6 per cent. These results are shown in Fig. 3 in which the per cent oxidation of ferritin sulfhydryl by completely aerobic liver slices is expressed as 100. Fig. 3 also shows the result of such pretreatment in nitrogen on the respiration (Q_{O_2}) of the normal rat liver

slices. Again, there is a progressive decrease of oxygen consumption concurrent with the reduced capacity to oxidize ferritin sulfhydryl.

Reversibility of Ferritin Sulfhydryl-Disulfide System by Liver Slices—In order to demonstrate the chemical transformation of the disulfide linkage in ferritin to the sulfhydryl form by liver slices (see Reaction B), experiments were carried out with ferritin (dialyzed against Ringer-phosphate,

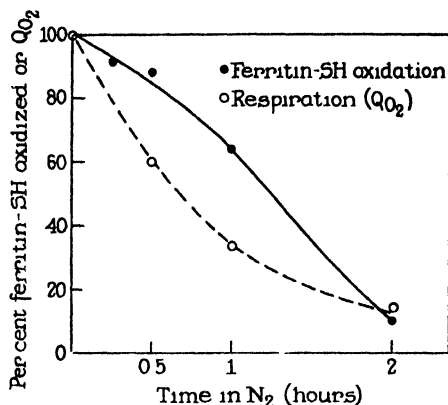


FIG. 3. Effect of prior incubation in nitrogen on the ability of normal rat liver slices to oxidize ferritin sulfhydryl groups and on the Q_{O_2} of the liver slices.

TABLE IV
Reversibility of Ferritin Sulfhydryl-Disulfide System by Liver Slices

Ferritin substrate	Incubation conditions	Ferritin SH content, μ M SH per 100 mg. ferritin N	
		Before incubation	After incubation
(a) Crystalline ferritin	No liver slices; 2 hrs. in O_2 or N_2	31.3	31.3
(b) " "	Rat liver slices; 2 hrs. in N_2	31.3	36.3
(c) " "	" " " 2 " " O_2	31.3	16.5
(d) Ferritin after treatment (c)	" " " 2 " " N_2	16.5	36.3

pH 7.4) as a substrate on rat liver slices; the sulfhydryl content of the ferritin was measured before and after incubation. A typical experiment is shown in Table IV. A solution of crystalline ferritin containing 31.3 μ M of sulfhydryl per 100 mg. of ferritin nitrogen was exposed to rat liver slices in an atmosphere of 100 per cent nitrogen for 2 hours at 37.5° (Table IV (b)). The sulfhydryl content of the ferritin rose to a value of 36.3. This increase of sulfhydryl groups is comparable to the increase previously noted after treatment of ferritin with reduced glutathione. In another

flask (Table IV (c)), the original ferritin solution was incubated with rat liver slices in an atmosphere of oxygen with a resulting decrease in ferritin sulfhydryl to 16.5 μM per 100 mg. of ferritin nitrogen, indicating an oxidation of ferritin sulfhydryl to ferritin disulfide. The ferritin solution obtained after aerobic incubation was then incubated with fresh rat liver slices in an atmosphere of nitrogen, with a resulting increase in sulfhydryl content to a value of 36.3 (Table IV (d)). This maximal level was the same as that obtained by anaerobic incubation of the original ferritin solution with rat liver slices. This experiment illustrates the reversibility of the oxidation-reduction of the ferritin sulfhydryl-disulfide system by liver slices in relation to the presence or absence of oxygen.

Activation and Inactivation of Vasodepressor Effect of Ferritin by Liver Slices in Vitro—Active ferritin is formed when normal liver slices are incubated in a Ringer-phosphate medium in 100 per cent nitrogen (Reaction B). Since the method of assay was the mesoappendix test, only the active form of ferritin (sulfhydryl ferritin) would be measured; it would be negative for any of the disulfide form. In order to estimate the relative amounts of each form of ferritin in the incubation medium, advantage was taken of the fact that the inactive or disulfide form is converted to the active sulfhydryl form after treatment with cysteine. A series of incubations was performed with rat liver slices incubated in 5 volumes of Ringer-phosphate, pH 7.4, at 37.5° for 2 hours in equilibrium with various oxygen-nitrogen mixtures. The incubation medium was centrifuged at 4700 r.p.m. and each supernatant divided into two aliquots. One was tested directly for vasodepressor activity. The other was first incubated with cysteine and then tested in a similar fashion. The results are given in Fig. 2 and are expressed in terms of the intensity of the vasodepressor response, from 0 to 4+. The solid portion of each column is a measure of the vasodepressor activity of the medium prior to treatment with cysteine and represents sulfhydryl ferritin. The total column (open + solid) is a measure of the vasodepressor activity after treatment of the medium with cysteine. Hence, the open portion of a column represents the disulfide form of ferritin originally present in the medium. At 100 to 60 per cent oxygen, no disulfide was present in the medium; it appeared first at 40 per cent. As the oxygen in the gas phase was diminished, the ratio of sulfhydryl to disulfide ferritin increased; in the absence of oxygen, all of the ferritin was in the sulfhydryl form. It should be noted that the first appearance of sulfhydryl ferritin in the untreated medium occurred at an oxygen concentration (10 per cent) corresponding to the point at which an abrupt and profound fall occurs in the capacity of the liver to oxidize the ferritin sulfhydryl groups.

Reactivation of Inactive Ferritin by Reduced Glutathione—Rat liver slices

were incubated with 5 volumes of ferritin-Ringer-phosphate for 3 hours in 100 per cent oxygen. A control ferritin solution incubated without tissue under the same conditions was found to contain $27.3 \mu\text{M}$ of sulfhydryl per 100 mg. of nitrogen. The ferritin, after exposure to liver slices, contained $8.4 \mu\text{M}$ of sulfhydryl per 100 mg. of nitrogen, indicating an oxidation of 69 per cent of the sulfhydryl groups as measured by iodoacetamide. The control ferritin solution gave a positive vasodepressor response at a dilution corresponding to 0.0003γ of ferritin nitrogen per 0.5 ml. injected into the test rat. The ferritin, after exposure to liver slices in oxygen, was only mildly active at 0.05γ of ferritin nitrogen per 0.5 ml., indicating a loss of about 99 per cent of the original ferritin activity. This contrasts with an oxidation of 69 per cent of the ferritin sulfhydryl groups. This is additional evidence that not all of the sulfhydryl groups which react with iodoacetamide are involved in the vasodepressor activity of ferritin.

An aliquot of the inactive ferritin, produced by oxidation with liver slices, was incubated for 30 minutes with reduced glutathione. The mixture was diluted and found to be active at 0.0005γ of ferritin nitrogen per 0.5 ml., representing a complete restoration of original activity. The quantity of glutathione used for reactivation was adjusted so that when the solution was diluted for testing the glutathione was well below the amount necessary to give a vasodepressor response (3).

Oxidation of Ferritin Sulfhydryl Groups by Cell-Free Liver Extract—The possibility was explored that the oxidation of ferritin sulfhydryl groups by liver slices in oxygen was mediated via a freely diffusible compound contained in the liver cell. Previous studies have shown that a cell-free extract from homogenized liver was capable of inactivating VDM activity aerobically (9). In the present study, cell-free extracts were prepared, without undue cellular damage, from surviving rat or rabbit liver by suspending slices in 5 volumes of Ringer-phosphate, pH 7.4, and incubating with shaking in an atmosphere of 100 per cent oxygen for 1 hour at 37.5° . The medium was separated from the slices and centrifuged for 10 minutes at 4700 r.p.m. in the cold centrifuge. Aliquots of the clear supernatant solution were mixed with ferritin at pH 7.4 and incubated at 37.5° in oxygen. Measurements of ferritin sulfhydryl groups before and after treatment with the liver slice extract revealed a progressive oxidation of sulfhydryl ferritin with time, up to a maximum at 2 hours. The total oxidation by the liver extract was in all cases lower by 15 to 20 per cent than that obtained with the intact liver slices. This oxidation was accompanied by loss of vasodepressor activity.

Quantitative Immunochemical Reactions of Sulfhydryl and Disulfide Ferritin—In a previous section, the total ferritin (sulfhydryl and disulfide forms) in dog liver extracts was determined by addition of antiferritin

serum which was standardized against a preparation of crystalline ferritin. It was necessary to ascertain whether an alteration in the ratio of sulfhydryl to disulfide groups in ferritin would modify the extent of combination of antibody with antigen. Aliquots of antiferritin serum were added to equivalent amounts of ferritin solutions which had been treated in a variety of ways to affect their sulfhydryl content. The specific precipitates obtained were treated in a manner previously described (4). Solution A was an untreated solution; Solution B was obtained by incubation of Solution A with rat liver slices in oxygen; Solution C was obtained by treating Solution A with 10 mg. of reduced glutathione; and Solution D,

TABLE V
Quantitative Precipitin Reaction of Ferritins with Varying Sulfhydryl Content

Ferritin	Ferritin SH content	Ferritin in N added to antiserum	Total N in ppt.	Per cent of control (A)
	<i>μm per 100 mg. N</i>	<i>mg.</i>	<i>mg.</i>	
A	27.3	0.026	0.222	100
		0.052	0.377	100
		0.104	0.637	100
		0.208	0.997	100
B	8.4	0.026	0.212	96
		0.052	0.380	101
		0.104	0.639	100
		0.208	0.990	99
C	35.0	0.026	0.215	97
		0.052	0.385	102
		0.104	0.642	101
		0.208	0.983	98
D	34.5	0.026	0.212	96
		0.052	0.368	98
		0.104	0.601	95
		0.208	0.964	97

by incubating Solution B with 10 mg. of reduced glutathione. Aliquots of Solutions A and B were treated directly with iodoacetamide for the estimation of their sulfhydryl groups. Solutions C and D were precipitated five times with equal volumes of saturated ammonium sulfate and the supernatants discarded each time in order to remove the glutathione. The final precipitates were dissolved in phosphate buffer, pH 7.4, and the sulfhydryl content estimated by reaction with iodoacetamide. The results are given in Table V. Within experimental error, the quantities of antigen-antibody complex precipitated by the various ferritins at several concentrations of ferritin nitrogen were the same. Amounts of ferritin and antiserum were chosen so as to provide an excess of antibody. Ferritins

ranging in sulfhydryl content from 8.4 to 35.0 μM per 100 mg. of ferritin nitrogen exhibited no immunochemical differences.

DISCUSSION

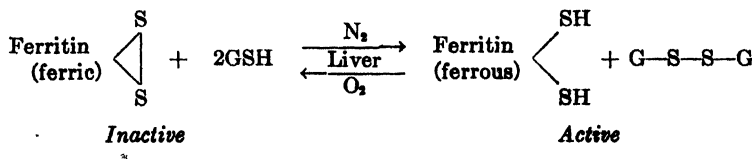
In vivo the counterpart to the anaerobic reduction of ferritin by liver slices occurs during the irreversible stage of hemorrhagic shock, in which there is a marked decrease in the portal venous oxygen saturation (10). That anaerobiosis is not the only stimulus for the production of reduced ferritin *in vivo* has become evident from our observations that active ferritin, together with a vasoexcitor material, is regularly present in the circulation of the renal hypertensive dog and in essential hypertension in man (11). This ferritin has been shown by catheterization of the hepatic vein to originate in the liver. Although no direct observations are available for the renal hypertensive dog, the hepatic blood flow and oxygen saturation have been found to be entirely normal in essential hypertension in man (12). A possible stimulus for the production of sulfhydryl ferritin by the aerobic liver in these hypertensive states may be the observed presence in the blood stream of the oppositely acting renal vasoexcitor material. In keeping with the appearance of active ferritin in the blood in the presence of a well oxygenated liver are our preliminary observations that the liver of the renal hypertensive dog has a much higher concentration of sulfhydryl ferritin than that of the normal animal (13 to 25 per cent of the total ferritin compared to 1 to 8 per cent in the normal).

From our experiments *in vivo* it would appear that ferritin in its active form can be released from the liver cell into the circulation and can be removed by inactivation by the well oxygenated liver. The experiments *in vitro* which showed the oxidation (inactivation) of ferritin sulfhydryl groups by liver were somewhat unphysiological, inasmuch as high concentrations of ferritin were incubated with liver slices, whose cellular permeability may be altered by the slicing technique and the nature of the incubation medium. One aspect of cellular activity was, however, unaffected by the high concentration of ferritin, namely the Q_{O_2} . Several explanations may be offered for the magnitude of the chemical alteration induced by the liver slices on this high concentration of ferritin. It is possible that under these conditions the chemical reaction between ferritin and the liver cells takes place in large measure on the surface of the cell. Another possibility is that the chemical alterations are mediated via a freely diffusible compound such as glutathione, which is constantly being oxidized and reduced by enzyme systems within the cell. Support for the latter explanation is provided by the demonstration that a cell-free extract from aerobic surviving liver slices is capable of inactivating the vasodepressor activity of ferritin, as well as of oxidizing ferritin sulfhydryl groups to the disulfide state.

Hellerman *et al.* have shown that not all of the sulfhydryl groups which can be measured in crystalline urease are involved in its enzymatic activity (13). This would also appear to be the case with respect to ferritin on the basis of two types of observations. Thus, a ferritin preparation, of which approximately 30 per cent of the sulfhydryl groups still remained after aerobic oxidation by liver slices, had lost all of its vasodepressor activity. It was also observed that dog muscle (cardiac, skeletal, and smooth) and kidney cortex, which are incapable of inactivating the vasodepressor property of ferritin, nevertheless oxidize some 7 to 16 per cent of the ferritin sulfhydryl groups, as measured with iodoacetamide.

One may calculate the number of molecules of the various forms of cystine sulfur per molecule of protein from the total cystine content of apoferritin (14), its molecular weight (15), and the sulfhydryl determinations (3). There are 65 molecules of total cystine sulfur in the protein molecule, of which a maximum of 26 molecules is in the sulfhydryl form as evidenced by their reactivity with iodoacetamide when the protein is in the native state. On denaturation, a total of 48 sulfhydryl molecules is available for reaction with iodoacetamide, leaving 17 molecules of sulfur presumably in the disulfide state and incapable of reduction by reduced glutathione when ferritin is in the native state. Since oxidation of but 70 per cent of the total sulfhydryl groups of native ferritin results in its complete inactivation, one may conclude that of the twenty-six sulfhydryl groups present in native ferritin a maximum of eighteen, and possibly less, is concerned in its activity.

Our finding that the sulfhydryl form of ferritin is the active vasodepressor, whereas the disulfide form is inactive, is consistent with all of our previous observations concerning the mode of activation and inactivation of this hepatic vasodepressor. These findings, together with the fact that glutathione can bring about these reactions *in vitro* and also reduce ferritin iron to the more labile ferrous state, are the basis for the following formulation.



SUMMARY

Normal aerobic dog liver contains ferritin almost entirely in the inactive disulfide state. Anaerobic incubation of the liver produces a progressive increase in the active sulfhydryl ferritin, together with an increase in reduced glutathione.

Liver slices in oxygen oxidize active sulfhydryl ferritin to the inactive

disulfide state. The extent of this reaction is a function of the oxygen tension. The inactivated disulfide ferritin can be reactivated with cysteine or reduced glutathione. Conversely, liver slices in the absence of oxygen convert disulfide ferritin to the active sulfhydryl form.

A cell-free solution, in which liver slices had been incubated aerobically, is also capable of inactivating the vasodepressor activity of ferritin under aerobic conditions. This inactivation is also accompanied by an oxidation of the sulfhydryl ferritin to the disulfide form.

It is postulated that the system within the liver cell which is concerned with the vasodepressor function of ferritin is dependent on an equilibrium between disulfide and sulfhydryl forms of ferritin. This equilibrium is affected by oxygen tension, as well as by other biological stimuli *in vivo*. A rôle for glutathione in this system is suggested.

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ANTAGONISTS OF NUCLEIC ACID DERIVATIVES

IV. REVERSAL STUDIES WITH 2-AMINOPURINE AND 2,6-DIAMINOPURINE

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Inhibition of the growth of *Lactobacillus casei* with folic acid as the nutrilit is a general property of 2,4-diaminopyrimidines, 2-aminopyrimidines, and condensed systems containing these groupings (1). These compounds comprise a spectrum of inhibitors. At one end lie the simpler diaminopyrimidines which are competitive antagonists of folic acid (1, 2). The center may be represented by 2-aminopurine (3), the action of which is reversible by either folic acid or a purine, while the inhibitory action of 2,6-diaminopurine (1, 3) is affected only slightly by the concentration of folic acid but is reversed by adenine.

The microbiological studies (1, 2) and many of the biological effects on higher animals are suggestive of an interference with nucleic acid synthesis. Thus diaminopurine has a protective effect in mouse leucemia (4), inhibits the hematopoietic tissues of dogs (5), swine (6), and chick embryo (7), inhibits the multiplication of vaccinia virus *in vitro* (8), and has a differential toxicity on cells of sarcoma 180 in tissue culture (9). The appearance of N¹⁵, administered as 2,6-diaminopurine, in the guanine fraction of the nucleic acid of rats (10) indicates the ability of diaminopurine to participate in nucleic acid metabolism, although it affords no clear picture of the metabolic pathways which are involved.

Studies on the specificity of the purine requirement of *L. casei* (11) have indicated that this organism possesses a mechanism for the interchangeable use of the physiological purines. Diaminopurine appears to interfere with this mechanism since at high concentrations its effects are reversed by adenine but not by guanine (1, 2), hypoxanthine, or xanthine. Reversal studies with free purines, nucleosides, and nucleotides reveal that the utilization of the ribose derivatives is inhibited more strongly than that of the free purines. The results of these studies, presented herein, suggest a number of inferences regarding the biochemical pathways in the nucleic acid metabolism of *L. casei*.

EXPERIMENTAL

The microbiological experiments were carried out as described previously (2). By the addition of supplements to the basal medium (O) the fol-

lowing media are obtained: OT, O + 1 γ of thymine per ml.; OFA, O + 0.046 m μ gm. of folic acid per ml.; PT, OT + 10 γ of adenine sulfate per ml. The cultures were incubated for 68 hours at 37°. Growth was estimated by titration of 10 ml. cultures with 0.1 N sodium hydroxide.

Results

When *L. casei* is grown on a folic acid medium without exogenous purine, as little as 0.1 γ of 2,6-diaminopurine per ml. abolishes growth (Fig. 1). At the lower levels of the inhibitor, the inhibition is reversible by folic acid; at higher levels of the inhibitor, complete reversal by folic acid is no longer possible.

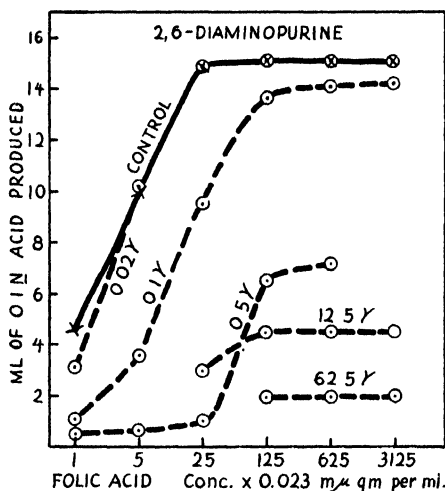


FIG. 1. Effect of folic acid on inhibitory action of 2,6-diaminopurine. Concentrations of 2,6-diaminopurine per ml. as indicated on the curves.

The inhibitory effect of 2-aminopurine in the folic acid medium is approximately 100th that of 2,6-diaminopurine (Fig. 2) and, at concentrations as high as 250 γ per ml., the inhibition is reversed by folic acid.

It had been previously found that the addition of adenine to the folic acid medium reverses the inhibitions produced by 2,6-diaminopurine and 2-aminopurine (1-3). When the other physiological purines were compared with adenine for their ability to reverse these inhibitions, some differences became apparent. At a level of 5 γ per ml., the inhibitory effect of diaminopurine is reversed by relatively low levels of adenine, guanine, and hypoxanthine (0.67 to 2 γ), but far less readily by xanthine (Fig. 3). At the 100 γ level of diaminopurine, on the other hand, the difference between adenine and the other natural purines as reversing

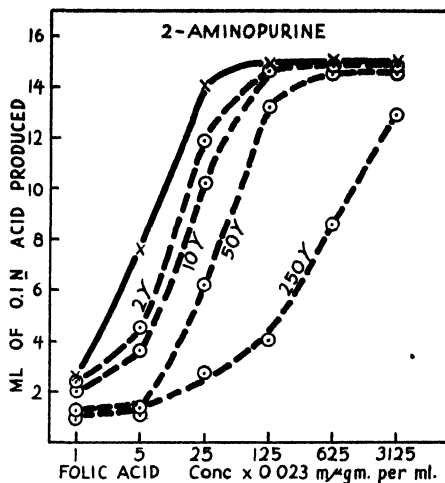


FIG. 2. Effect of folic acid on inhibitory action of 2-aminopurine. Concentrations of 2-aminopurine per ml. as indicated on the curves.

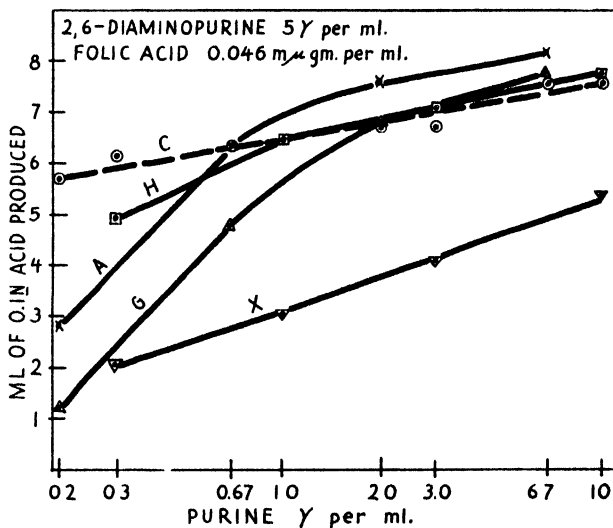


FIG. 3. Effect of purines on inhibitory action of 2,6-diaminopurine. Curve C, control without diaminopurine; Curve H, hypoxanthine; Curve A, adenine; Curve G, guanine; Curve X, xanthine.

agents is very pronounced (Fig. 4). Whereas 2 γ of adenine restore the titer to almost 90 per cent of the control, guanine and hypoxanthine produce only a slight reversal and xanthine none at all, even at a concentration of 10 γ per ml.

The amount of adenine required to restore full growth in this experiment approximates the amount necessary for growth in the thymine-containing medium (Fig. 6 below). Therefore, tests at higher levels of the inhibitor¹ were required to rule out the possibility of a non-competitive

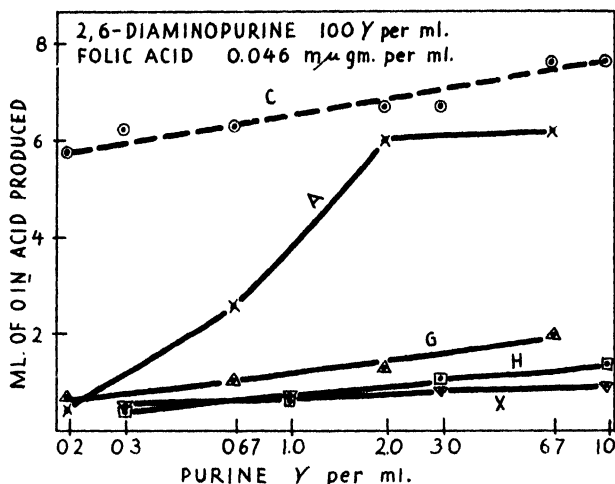


FIG. 4. Effect of purines on inhibitory action of 2,6-diaminopurine. Curve C, control without diaminopurine; Curve A, adenine; Curve G, guanine; Curve H, hypoxanthine; Curve X, xanthine.

TABLE I
Effect of Adenine on Inhibitory Action of 2,6-Diaminopurine in OFA Medium

Adenine sulfate mg. per 10 ml.	2,6-Diaminopurine, mg. per 10 ml.		
	0	3	10
	Acid production, ml. 0.1 N acid per 10 ml.		
0	5.8	0.6	0.7
0.1	7.1	2.0	0.9
0.3	7.5	1.8	0.8
1.0	7.6	1.7	0.8

type of inhibition. The results of these experiments (Table I) show that at 300 γ of diaminopurine per ml. reversal by adenine is only partial.

The inhibition produced by 30 γ per ml. of 2-aminopurine in the folic acid medium is readily reversed by 1 to 3 γ of the four physiological pu-

¹ High concentrations of both adenine and 2,6-diaminopurine may be achieved by use of the purine lactates (*cf.* (12)).

rines, although the reversal by xanthine is only 80 per cent complete. At 500 γ per ml. (Fig. 5) xanthine is ineffective, but the other purines are capable of a considerable restoration of growth.

Since *L. casei* can grow in a medium containing thymine and one of the natural purines (11, 13, 14) in lieu of folic acid, the effect of diaminopurine in this medium also was investigated. When adenine is the purine added to the thymine medium, low concentrations of diaminopurine appear to be stimulatory, whereas concentrations above 100 γ per ml. are inhibitory (Fig. 6). When any of the other physiological purines is used as the metabolite in place of adenine, the inhibitory effect of diaminopurine

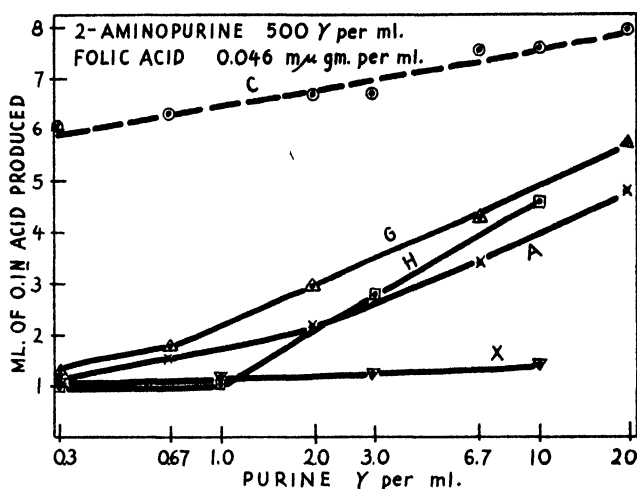


FIG. 5. Effect of purines on inhibitory action of 2-aminopurine. Curve C, control without 2-aminopurine; Curve G, guanine; Curve H, hypoxanthine; Curve A, adenine; Curve X, xanthine.

is more pronounced (Fig. 7). Thus, at a level of 50 γ per ml. of diaminopurine, there are no inhibition in the presence of adenine, only partial growth with guanine, and no growth at all with xanthine and hypoxanthine. The level of diaminopurine required for inhibition appears to be greater than in the folic acid-containing medium (cf. Fig. 4). This difference is scarcely perceptible, however, when growth in the two media is compared in the presence of the same level of adenine. Nevertheless, reversal occurs at higher levels of diaminopurine in the medium which contains thymine.

Although a concentration of 100 γ of 2-aminopurine shows no inhibition of growth in the PT medium, when the added purine is adenine, in the usual 3 day assay period (1) an extension of the assay time to 14 days

reveals such an inhibition even in the presence of adenine (Fig. 8). This may reflect an interference with glycolysis rather than with growth. In

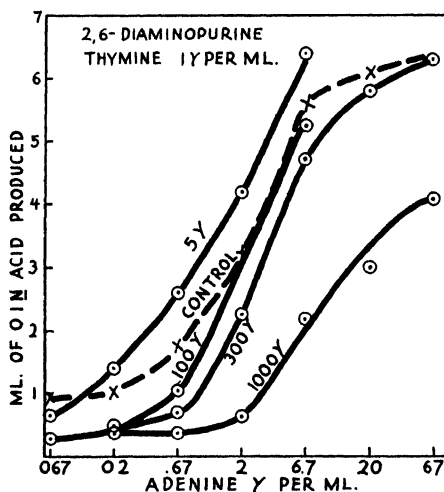


Fig. 6. Effect of adenine on inhibitory action of 2,6-diaminopurine. Concentrations of 2,6-diaminopurine per ml. as indicated on the curves.

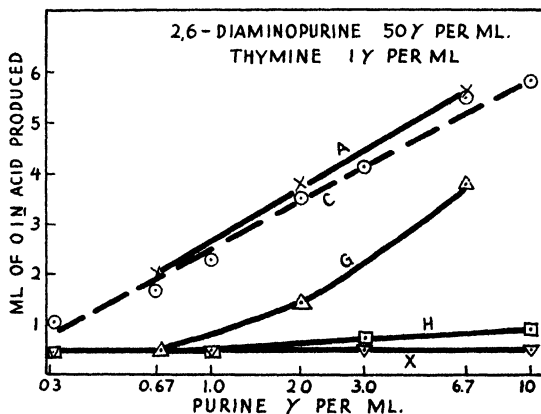


Fig. 7. Effect of purines on inhibitory action of 2,6-diaminopurine. Curve C, control without diaminopurine; Curve A, adenine; Curve G, guanine; Curve H, hypoxanthine; Curve X, xanthine.

a similar time experiment with diaminopurine, no such inhibition was manifested when adenine was present.

When the level of 2-aminopurine is raised to 500 γ per ml., it is possible to see inhibitory effects in the usual assay time, even in the presence of adenine. The difference in the effectiveness of the four purines in reversing

this inhibition follows the order: adenine > guanine > hypoxanthine > xanthine (Fig. 9). This may be contrasted with the results in the OFA medium (Fig. 5) where guanine and, at some concentrations, hypoxanthine appear to be more effective than adenine as reversing agents.

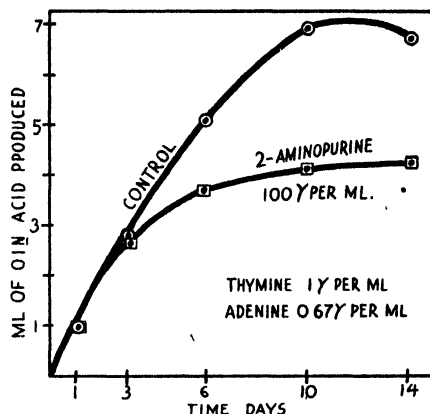


FIG. 8. Effect of 2-aminopurine on acid production over an extended period of time.

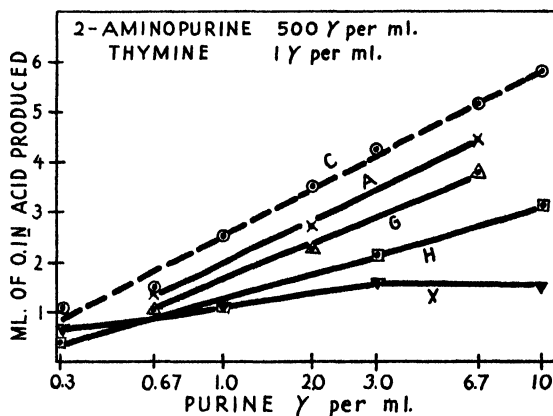


FIG. 9. Effect of purines on inhibitory action of 2-aminopurine. Curve C, control without 2-aminopurine; Curve A, adenine; Curve G, guanine; Curve H, hypoxanthine; Curve X, xanthine.

Two unnatural purines were examined for their ability to reverse the inhibitory effects of diaminopurine (Table II). These are 6-methylaminopurine, which had shown purine-like activity in promoting the growth of *L. casei* with thymine (11), and 7-methyladenine, which had been inactive in this respect (11). As was to be expected, 6-methylaminopurine is

about 1 per cent as effective as adenine in reversing the effect of 0.1 mg. of diaminopurine but cannot replace adenine as a reversing agent when the 1 mg. level of diaminopurine is used, whereas 7-methyladenine has no purine-like activity as a diaminopurine antagonist.

Ribonucleosides and nucleotides were found in previous work (11) to be somewhat inferior to free purines on a molar basis in the growth of *L. casei* with thymine, and it appeared, therefore, that degradation to free purines probably precedes utilization. It was of some interest, however, to test these substances for possible effects in reversal of the inhibitory action of the unnatural purines. It was found that the utilization of the ribose derivatives is inhibited in the presence of diaminopurine (Table III). Thus, for example, adenosine at a concentration of 0.12 mg. per 10 ml. has growth-promoting ability in the OT medium approximately equivalent to that of 0.02 mg. of free adenine (Fig. 6, control Curve X,

TABLE II
Reversals of Effect of 2,6-Diaminopurine on L. casei in OFA Medium

Compound	Concentration, mg. per 10 ml	2,6-Diaminopurine, mg. per 10 ml.	Acid production, ml. 0.1 N acid per 10 ml.
6-Methylaminopurine hydrochloride	0.1	0	6.8
		0.1	0.55
		1.0	0.55
		0.1	0.9
		1.0	8.4
		0.1	0.4
7-Methyladenine	1.0	1.0	1.1
	1.0	0.1	0.45
	1.0	1.0	0.25

adenine 2 γ per ml.). In the presence of 0.05 mg. of diaminopurine, however, the growth with adenosine was abolished, whereas that with adenine was increased. Similarly, the utilization of other ribonucleotides and nucleosides was markedly inhibited by diaminopurine. The ability of these substances at high levels to reverse the action of diaminopurine might be explained if 1 or 2 per cent of free purines as impurities was present, amounts generally within the limits found by Carter (17) in commercial preparations. Guanylic acid, however, appeared to be somewhat exceptional, since this substance was about as effective as guanine on a molar basis (cf. Fig. 7). This substance, consequently, was selected for more thorough study.

Commercial guanylic acid was treated by the paper chromatographic technique of Hotchkiss (16). The papers were dried, cut into measured lengths, and substituted for the purine supplement in the *L. casei* medium containing 0.05 mg. of diaminopurine per 10 ml. This confirmed the

presence of an impurity, possibly hypoxanthine, capable of reversing the action of diaminopurine. However, the original spot (guanylic acid does not migrate) had a higher order of activity than other nucleosides and nucleotides. Since guanine itself does not migrate by this technique, this activity may have been due to free guanine. It was then found that

TABLE III
*Effect of Nucleosides and Nucleotides on Action of
2,6-Diaminopurine and 2-Aminopurine*

Substance*	Concentration, mg. per 10 ml	OFA medium					OT medium				
		No in- hib- itor	2,6-Diaminopurine, mg. per 10 ml.			2-Aminopurine, 5 mg. per 10 ml.	No in- hib- itor	2,6-Diaminopurine, mg. per 10 ml.			2-Aminopurine, 5 mg. per 10 ml.
			0.05	0.25	1.0			0.05	0.25	1.0	
Acid production, ml. of 0.1 N acid per 10 ml.											
Control		6.0	0.6	0.6	0.4	0.6	0.4	0.4	0.4	0.4	0.4
Adenosine	0.12	6.0	1.4				3.7	0.5			
	0.48	6.1	5.4	4.8	0.9	1.8	7.6	2.5	1.4	0.7	1.4
Guanosine	0.48	6.7	4.1	0.8		1.0	5.0	2.6	1.1		1.0
Xanthosine	0.48	5.9	1.7	0.9		1.6	2.8	2.0	0.9		1.3
Inosine	0.62	6.4	4.7	1.8		1.6	3.4	2.6	1.3		1.2
Adenosine-3'-phosphoric acid	0.64	6.2	5.1	5.1	1.4	2.1	7.3	4.5	3.0	0.8	2.0
Guanylic acid	0.64	6.0			3.5	5.8	5.9			2.6	3.7
Same, purified	0.64	6.4	6.4	2.8	0.9	3.0	6.3	3.2	1.7	0.6	1.5
Xanthylic acid	0.50	5.6	5.1	2.4		2.0	3.2	2.6	1.3		1.2
Inosine-3'-phosphoric acid	0.64	5.4	1.2	1.3		2.9	2.5	1.3	1.2		1.9

* The substances listed are of commercial origin or were prepared in these laboratories by standard methods (15) and were pure by analytical findings, absorption spectra (16), and paper chromatography (16). That these criteria may not be adequate is illustrated with respect to guanylic acid (see the text). The nucleotides of adenine and hypoxanthine are designated as 3'-phosphoric acids to distinguish them from the corresponding 5'-phosphoric acids but without regard to *a* and *b* isomerism (*cf.* (17)). As prepared by the usual methods, the nucleotides from yeast nucleic acid are largely of the 3' variety (C. E. Carter and W. E. Cohn, unpublished remarks).

guanylic acid free from purine bases or nucleosides could be prepared through an adaptation of the anion-exchange separation of Cohn (18).²

² 200 mg. of commercial guanylic acid were neutralized with ammonia and the solution filtered. It was adsorbed on a column (12 mm. × 60 mm.) of Dowex-1-A, washed with 25 ml. of water and 50 ml. of 0.002 N HCl. Elution with 0.1 N HCl followed. The first 3 ml. of 0.1 N HCl contained guanylic acid and some unidentified impurity, as evidenced by some deviations in the absorption spectrum from that of guanylic acid. The next 25 ml. of eluate contained about 95 per cent of the guanylic acid in apparently pure form.

This purified material was much less active as a reversing agent.³ It may nevertheless be true that guanylic acid is somewhat more active than the other nucleotides, but this activity in any case is of a low order of magnitude.

The results of similar studies on the effects of nucleotides and nucleosides in the presence of 2-aminopurine also are shown in Table III. This substance, like 2,6-diaminopurine, appears to interfere with the utilization of the nucleosides and nucleotides.

DISCUSSION

The two inhibitory purines, 2-aminopurine and 2,6-diaminopurine, are similar in that both appear to interfere with the utilization of exogenous purine ribonucleosides and nucleotides. However, the action of 2-aminopurine is readily reversed by folic acid, while restoration of growth by this metabolite can take place only with the lower concentrations of 2,6-diaminopurine. Furthermore, with 2-aminopurine, guanine, hypoxanthine, and adenine are all effective in the reversal of the inhibitions, whereas with 2,6-diaminopurine a high degree of specificity is shown by adenine.

The amount of adenine required for maximal growth in the thymine-containing medium is approximately 6.7 γ per ml. (Fig. 6) and at this level the adenine is converted essentially quantitatively into polynucleotide purine⁴ (19). In the folic acid system, inhibited by diaminopurine, equivalent growth is produced by one-tenth this amount of adenine (Fig. 3). This suggests that a source of purine, in addition to the added adenine, is available to the growing organism. This might imply the restoration of a function of the folic acid system which was blocked by the inhibitor. However, the utilization of some diaminopurine for growth purposes would explain this finding and also the stimulation of growth which is observed at the lower levels of diaminopurine in the thymine-adenine system (Fig. 6) (10, 20).

The observation that diaminopurine interferes with the utilization of purine ribosides and ribonucleotides by *L. casei* suggests the possibility that its inhibitory action on growth is the result of this effect. Thus, if the synthesis of the purine ring, as mediated by the folic acid system, proceeds via an intermediary ribose phosphate (21), diaminopurine might block the utilization of the nucleotide which is the primary product of

³ Presumably, the activities of all the ribose derivatives shown in Table III should be regarded as maximal and subject to revision downward after further purification of the compounds. The essential finding that the activities of the ribose derivatives relative to the free purines are much diminished in the presence of 2,6-diaminopurine would remain unaltered.

⁴ Hitchings, G. H., Light, A., Lorz, D., and VanderWerff, H., to be published.

purine synthesis. Similarly, the interconversion of exogenous purines would be blocked if these reactions proceed via ribosides. An alternative mechanism for the utilization of free adenine would be required to explain the ability of this substance to restore growth in the presence of diaminopurine.

The authors are indebted to Henry VanderWerff for microbiological assays.

SUMMARY

Both 2-aminopurine and 2,6-diaminopurine inhibit the growth of *Lactobacillus casei* more strongly in a folic acid than in a thymine-containing medium. Both block the utilization of purine ribosides and ribonucleotides more effectively than that of the free purines.

The inhibitory action of 2-aminopurine is reversed either by folic acid or by purines over a considerable range of concentration. In a medium containing folic acid, growth is restored essentially equally well by adenine, guanine, or hypoxanthine, but xanthine is almost inactive. In the medium containing thymine, adenine and guanine give better growth than hypoxanthine.

Reversal of the inhibitory effects of 2,6-diaminopurine by folic acid takes place only at low concentrations of the inhibitor. At low concentrations of diaminopurine, growth is restored by adenine, guanine, and hypoxanthine, with xanthine less active. At high concentrations of the inhibitor, only adenine can restore growth. In the medium containing thymine, adenine is much more effective than other purines in the restoration of growth, but guanine has activity which is greater than that shown in the folic acid-containing medium.

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THE COLORIMETRIC DETERMINATION OF THIOCYANATE IN WHOLE BLOOD

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No method is available for the practical and accurate determination of thiocyanate in whole blood. The exact concentration of thiocyanate in whole blood may, at times, be of interest in clinical studies employing thiocyanate, in studies on the "thiocyanate space," and in other biochemical, pharmacological, and toxicological researches on thiocyanate. An obvious advantage of analyzing whole blood is the small amount of blood needed for analysis.

The method to be described, similar to other known colorimetric procedures for thiocyanate determination in biological fluids (1, 2), depends essentially on the photoelectric measurement of light absorption by ferric thiocyanate. The photoelectric determination of thiocyanate as ferric thiocyanate can only be performed on clear protein-free filtrates. When trichloroacetic acid, the precipitant employed in all reported methods of thiocyanate determination in plasma (serum), is used to precipitate the whole blood proteins, only a small fraction of the thiocyanate present in blood appears in the filtrate. This was first observed qualitatively by Gregersen and Stewart (3). Quantitative studies in this laboratory¹ revealed that 60 to 90 per cent of the sodium thiocyanate added to blood, in amounts corresponding to therapeutic and toxic concentrations found in patients, could not be recovered from trichloroacetic acid filtrates of whole blood. When the blood was hemolyzed before the addition of the precipitant, recovery was about twice as high but did not exceed 70 per cent. An inverse proportionality between hemoglobin concentration and thiocyanate recovery was noted, pointing to hemoglobin as the factor responsible for the loss of thiocyanate. Some experimental evidence was obtained which indicated that the loss of thiocyanate was caused by adsorption of the anion on hemoglobin, facilitated by the reversal of the charge on the hemoglobin molecules after the addition of the first few drops of the strongly dissociated trichloroacetic acid. The foregoing observations seem to be of practical importance, since it is not impossible that determinations of thiocyanate in whole blood may have occasionally been employed clinically and may perhaps account for some of the dis-

¹ Goldstein, F., unpublished data.

crepancies noted by some investigators (4) between apparently low blood thiocyanate levels on the one hand and high dosages and severe toxic symptoms on the other hand.

Obviously, trichloroacetic acid cannot be employed in the quantitative determination of thiocyanate in whole blood. Other acid precipitants are not likely to be useful either. The use of ethyl alcohol has been adopted in this work and has proved to be completely satisfactory.

Methods

Reagents—

1. 95 per cent ethyl alcohol.
2. Ferric nitrate reagent. 50 gm. of ferric nitrate crystals are dissolved in about 500 ml. of distilled water, 525 ml. of concentrated nitric acid are added, and the volume of the solution is brought to 2 liters with distilled water. The strongly acid solution is necessary to prevent hydrolysis of the ferric salt after mixing with the alcoholic filtrate. The reagent remains stable for several months.
3. Standard thiocyanate solution. A 0.02 per cent aqueous solution of sodium thiocyanate. The solution is stable but is preferably made up fresh once a month to prevent changes in concentration due to evaporation.

Procedure.

To 1 ml. of heparinized blood in a large test-tube (35 to 50 ml.) is added 1 ml. of distilled water to compensate for the 1 ml. of aqueous sodium thiocyanate solution to be added to the standard sample. The proteins are precipitated by the slow addition of 25 ml. of 95 per cent ethyl alcohol. The test-tube with its contents is shaken vigorously while the first 10 ml. of alcohol are delivered. After all the alcohol is added, the tube is inverted once or twice. The mixture is allowed to stand for 2 minutes and, after the test-tube again is shaken, filtered through Whatman No. 2 filter paper. 10 ml. of the filtrate are pipetted into a test-tube and 1 ml. of the ferric nitrate reagent is added under dimmed light. The color develops immediately. The solution is allowed to stand for 5 minutes, during which time a sometimes marked turbidity develops. This is apparently owing to the presence in the filtrate of traces of protein which are precipitated by nitric acid. The solution is again filtered through Whatman No. 2 filter paper (in dimmed light) and 5 ml. of the filtrate are directly collected into a Klett colorimeter tube. The density of the solution is then determined in the Klett-Summerson photoelectric colorimeter. A Klett No. 52 filter is used. After the addition of the ferric nitrate reagent, the entire procedure is carried out in dimmed light.

Calculation of Results

The results are calculated in the usual way by reference to the reading of a standard or by using a calibration curve. A calibration curve is easily prepared by adding graded amounts of sodium thiocyanate to 1 ml. portions of identical blood and carrying out the procedure as outlined above (Fig. 1). A control blood filtrate is prepared in the same way and the colorimeter reading of the control is subtracted from the readings of the standard samples. If a standard is used, it should be prepared by adding 1 ml. of 0.02 per cent NaSCN solution to 1 ml. of thiocyanate-free blood and carrying out the procedure outlined. A control should

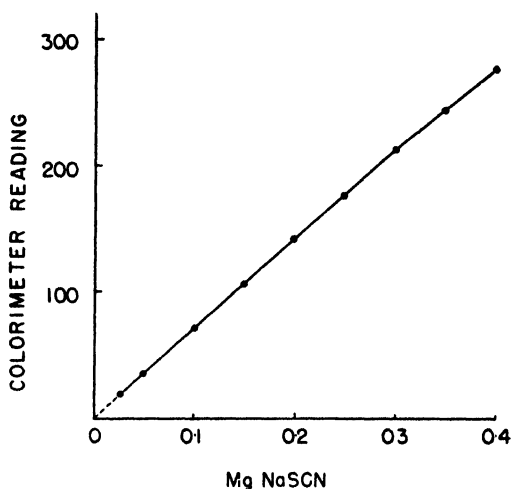


FIG. 1. Color-concentration curve (calibration curve) showing the relation between the amount of thiocyanate present in 1 ml. blood samples and readings on the Klett-Summerson photoelectric colorimeter.

be prepared from thiocyanate-free blood and the reading subtracted from both the standard and the unknown. The control readings of various blood samples not containing thiocyanate above normal low levels are fairly constant and should be determined for each instrument. The mean value found could be used in routine work as a constant to be subtracted from standard and unknown readings.

Stability of Color

Since in this procedure the colorimeter readings are taken about 10 minutes after the development of the color, it is important that during that time no fading of color should occur. The stability of the color of ferric thiocyanate solutions was tested in a series of experiments. Both

bright daylight and artificial light caused rapid fading of the color to the extent of approximately 25 per cent of the original intensity within 1 hour. When the solutions were covered with a towel or a piece of cloth providing dimmed light, no fall in the colorimetric reading occurred within 1 hour. One sample was allowed to stand in a darkened spot for 48 hours and after that time showed a reduction in light absorption of only 3 per cent.

Accuracy of Method

The color reaction on which this method depends is well known and its applicability to the quantitative determination of thiocyanate need not be discussed. The data contained in Table I and Fig. 1 indicate that

TABLE I
Recovery of Sodium Thiocyanate from 1 Ml. Blood Samples

Specimen No.	Amount of NaSCN added	Recovery of NaSCN
	mg.	per cent
1	0.2	100.5
2	0.3	100.3
3	0.1	99.0
4	0.3	100.3
5	0.35	98.0
6	0.4	97.5
7	0.1	100.0
8	0.2	99.5
9	0.05	101.8
10	0.15	101.3
11	0.25	100.4
12	0.025	97.1

the method as described here yields accurate results within a limited range of concentrations. Recovery of added thiocyanate ranged between 97.1 and 101.8 per cent, the mean recovery being 99.6 per cent. The color-concentration curve is a straight line until colorimeter readings of about 250 are reached, and the curve can be extrapolated back to zero. With the proportions of reagents and blood used in these experiments optimal results are obtainable with concentrations of sodium thiocyanate ranging between 2.5 and 40 mg. per 100 ml. of blood. If higher (or lower) concentrations are expected, 0.5 ml. (or 2 ml.) of blood may be used for analysis, as well as for the preparation of the control and standard, with the amounts of reagents unchanged.

The subtraction of the control reading (as indicated above) is important because in the spectral region where ferric thiocyanate can be measured

the reagents alone, as well as chloride and certain chromogens always present in blood filtrates, cause an appreciable light absorption. The light filter used here, Klett No. 52, was chosen because the light transmitted by this filter is only absorbed to a small extent by ferric chloride and is sufficiently well absorbed by ferric thiocyanate. An alternate, even superior, filter for the measurement of ferric thiocyanate was found in a combination of Wratten No. 45 and No. 58 filters.

SUMMARY

A method is described for the accurate determination of thiocyanate in whole blood. No other method for this purpose has been available heretofore. The procedure described is a modification of existing methods of colorimetric determination of thiocyanate in biological fluids. Trichloroacetic acid, generally used as the protein precipitant but unsuitable for use in the determination of thiocyanate in whole blood, has been replaced by 95 per cent ethyl alcohol. Minor modifications, necessitated by the use of ethyl alcohol, have been introduced.

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THE LIPOTROPIC EFFECT OF FOLIC ACID ON RATS RECEIVING VARIOUS PURIFIED DIETS*

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In previous experiments in our laboratories tissue sections of the livers of rats receiving a diet containing 10 per cent glycine showed evidence of a fatty infiltration which was not present when the animals were given folic acid in addition to the high level of glycine. There is usually a marked increase in neutral fat with only a slight increase in total fatty acids in livers of animals which show evidence of a fatty infiltration. It was thought that glycine might in some manner alter the distribution of the fatty acids in the livers of these animals.

In this paper are reported the results of cholesterol, total fatty acid, and phospholipide determinations on livers of rats which had received various purified diets containing no added folic acid, as compared with animals which had received these same diets supplemented with folic acid or vitamin B₁₂ or both.

EXPERIMENTAL

Weanling litter mate rats of the Sprague-Dawley strain weighing 45 to 60 gm. were used in these experiments. The basal diet consisted of Labco casein 18 gm., sucrose 68 gm., hydrogenated vegetable oil 10 gm., cod liver oil 2 gm., and salt mixture (1) 2 gm. To each kilo of diet were added inositol 1 gm., choline chloride 1 gm., thiamine chloride 5 mg., riboflavin 5 mg., pyridoxine chloride 5 mg., calcium pantothenate 10 mg., nicotinic acid 20 mg., menadione 0.25 mg., and biotin 0.024 mg. The various modifications of this diet which were employed are shown in Table I. The glycine and ribonucleic acid replaced an equal amount of sucrose when added to the diet. The commercial ribonucleic acid which was used was dissolved in alkali and reprecipitated to remove any folic acid which might have been present as a contaminant. The growth data for the various groups are also presented in Table I.

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TABLE I

*Summary of Diet Compositions and Weight Gains of Animals
Receiving Purified Diets with Various Supplements*

The glycine and ribonucleic acid (RNA) when added replaced an equal amount of sucrose. The folic acid was added at a level of 5 mg. per kilo of diet and the vitamin B₁₂ (Squibb's rubramin) was added at a level of 50 γ per kilo of diet.

Group No.	Average initial weight (No. of animals)	Supplement to basal diet	Average total gain in weight during 45 day experimental period
	gm.		gm.
I	53 (8)	None	208 $\pm$ 6.6*
II	54 (8)	Folic acid	224 $\pm$ 4.6
III	51 (7)	10% glycine	114 $\pm$ 10.0
IV	55 (8)	10% " + folic acid	174 $\pm$ 3.9
V	53 (6)	10% " + vitamin B ₁₂	127 $\pm$ 4.9
VI	54 (7)	10% " + 4% RNA	99 $\pm$ 7.1
VII	55 (8)	10% " + 4% " + vitamin B ₁₂	107 $\pm$ 4.3
VIII	55 (8)	10% " + 4% " + folic acid + vitamin B ₁₂	181 $\pm$ 4.4

* Standard error.

TABLE II

Effect of Folic Acid on Total Fatty Acid Content of Livers of Rats Receiving Various Purified Diets

Diet*	No. of animals	Total fatty acid in liver	
		per cent	mg.
Basal	5	3.92 $\pm$ 0.29†	229
" + 10% glycine	10	5.67 $\pm$ 0.44	346
" + 10% " + 500 γ folic acid per 100 gm.	10	3.63 $\pm$ 0.22	287
Basal + 10% glycine + 2% RNA	5	9.01 $\pm$ 1.26	716
" + 10% " + 2% " + 500 γ folic acid per 100 gm.	4	3.97 $\pm$ 0.13	292

* The animals received the experimental diets for 60 days. The glycine and ribonucleic acid (RNA) when added replaced an equal amount of sucrose.

† Standard error.

Methods

Two different methods were used for the determination of the total fatty acids. In a preliminary experiment, the results of which are shown in Table II, the method employed was that of van de Kamer *et al.* (2) which consists of saponification of the liver with alcoholic KOH, followed by neutralization with HCl, extraction of the fatty acids with petroleum ether,

and subsequent titration of the fatty acids with 0.1 N NaOH. The amount of fatty acid present was then calculated, assuming an average molecular weight of 284 for the fatty acids. In the subsequent experiments, when the various fractions of the fats were determined separately, the procedures outlined by Bloor (3-4) were used, the cholesterol being determined by the Liebermann-Burchard H_2SO_4 -acetic anhydride color reaction and the total fatty acids and phospholipide by the silver dichromate oxidation procedure. Duplicate or triplicate determinations were carried out on each liver.

TABLE III

Effect of Folic Acid on Cholesterol and Total Fatty Acid Content of Livers of Rats Receiving Various Purified Diets

Group No.	Diet*	No. of rats†	Cholesterol in liver		Total fatty acids in liver	
			per cent	mg.	per cent	mg.
I	Basal	4	$0.86 \pm 0.12\ddagger$	83	$3.43 \pm 0.53\ddagger$	311
II	I + folic acid	5	0.79 ± 0.15	86	1.85 ± 0.60	198
III*	" + glycine	4	0.67 ± 0.10	54	3.28 ± 0.42	275
IV	III + folic acid	4	0.63 ± 0.08	67	1.77 ± 0.33	192
V	" + vitamin B ₁₂	4	0.68 ± 0.07	65	2.78 ± 0.61	271
VI	" + RNA	4	1.03 ± 0.06	99	8.17 ± 2.06	801
VII	VI + vitamin B ₁₂	5	1.32 ± 0.18	122	8.65 ± 1.24	783
VIII	VII + folic acid	5	0.65 ± 0.06	58	2.18 ± 0.62	180

* See Table I.

† Duplicate or triplicate determinations were carried out on each liver.

‡ Standard error.

DISCUSSION AND RESULTS

The diet composition and results of a preliminary experiment in which only total fatty acids were determined are given in Table II. In these groups of animals, which had been on the experimental diets for 60 days, the inclusion of 10 per cent glycine in the diet gave rise to a statistically significant ($P = 0.02$) increase in the total fatty acids over that found with the basal diet. The addition of folic acid prevented this increase in fatty acids. The addition of 2 per cent ribonucleic acid to the high glycine diet caused an even greater increase in liver fatty acids, and, in fact, the increase over that on the high glycine diets alone was highly significant ($P = 0.01$). Again the addition of folic acid to the diet prevented this increase.

The results of a similar experiment, in which the animals were maintained on the experimental diets for only 45 days, are given in Tables III, IV, and V. The diet composition and weight gains for these groups are presented in Table I. Table III gives the values obtained for cholesterol

and it will be noted that there are no significant differences among the first five groups. The values for those animals which received the high glycine diets do appear to be somewhat lower than those for animals receiving the basal diet alone. The values for Groups VI and VII, which received 4 per cent ribonucleic acid in addition to the glycine, are significantly increased ($P = 0.02$) and the addition of folic acid (Group VIII) prevented this increase. The values for phospholipide are given in Table IV. The values for fatty acid present as phospholipide were calculated on the basis of oleyl-stearyl lecithin. Although the differences among the various groups are not statistically significant, the animals receiving the high level of glycine tended to have a lower liver phospholipide and in every case

TABLE IV
Effect of Folic Acid on Liver Phospholipides of Rats Receiving Various Purified Diets

Group No.	Diet*	No. of rats†	Phospholipide in liver		Liver phospholipide fatty acids‡	
			per cent	mg.	per cent	mg.
I	Basal	4	2.13 $\pm$ 0.34§	217	1.50 $\pm$ 0.24§	153
II	I + folic acid	5	2.28 $\pm$ 0.10	252	1.60 $\pm$ 0.22	178
III	" + glycine	4	1.52 $\pm$ 0.47	126	1.07 $\pm$ 0.33	89
IV	III + folic acid	4	2.17 $\pm$ 0.57	230	1.53 $\pm$ 0.40	164
V	" + vitamin B ₁₂	4	2.22 $\pm$ 0.56	222	1.56 $\pm$ 0.39	168
VI	" + RNA	4	1.96 $\pm$ 0.29	185	1.38 $\pm$ 0.20	130
VII	VI + vitamin B ₁₂	5	1.10 $\pm$ 0.85	100	0.78 $\pm$ 0.22	70
VIII	VII + folic acid	5	2.46 $\pm$ 0.39	218	1.73 $\pm$ 0.85	155

* See Table I.

† Duplicate or triplicate determinations were carried out on each liver.

‡ Calculated on the basis of oleyl-stearyl lecithin.

§ Standard error.

in which folic acid was added to the diet there was a tendency toward a slightly higher value for liver phospholipide.

The values for total fatty acids are given in Table III. The addition of folic acid to the basal diet, to the 10 per cent glycine diet, and to the diet containing 10 per cent glycine and ribonucleic acid brought about, in every case, a decrease in total fatty acids which was either statistically significant or bordered on statistical significance.

Although glycine did not increase the total fatty acid in the second experiment, there was an alteration in the distribution of liver fatty acids, as can be seen from Table V. One possible explanation for this apparent discrepancy with the results reported in Table II is that the animals in the second experiment received the experimental diets for only 45 days and a longer period may be required for the production of an actual increase in total fatty acids.

Table V also shows the values obtained for neutral fat. These values were obtained by subtracting the average value for phospholipide fatty acids from the average value for total fatty acids for the various groups. It can be readily seen from these data that the predominant lipotropic effect of folic acid is exerted on the neutral fat fraction of the liver fatty acids.

The inclusion of vitamin B₁₂ in the diets brought about little alteration in any of the various fat fractions. However, all of the animals received a diet containing 18 per cent casein, and, since this amount of casein probably contains sufficient vitamin B₁₂ to meet the needs of the rat, additional amounts of vitamin B₁₂ might be expected to have no appreciable effect on the liver fats of these animals.

TABLE V
*Effect of Folic Acid on Fatty Acid Content of Liver Neutral Fat of Rats
Receiving Various Purified Diets*

Group No.	Diets*	Liver neutral fat fatty acids	
		per cent	mg.
I	Basal	1.57	158
II	I + folic acid	0.25	20
III	" + glycine	2.21	186
IV	III + folic acid	0.24	29
V	" + vitamin B ₁₂	1.22	103
VI	" + RNA	6.79	671
VII	VI + vitamin B ₁₂	7.87	713
VIII	VII + folic acid	0.45	25

* See Table I.

No explanation can be given at present for the antilipotropic effect exerted by the ribonucleic acid. The data obtained with the diets containing ribonucleic acid are included merely as further evidence of the lipotropic effect of the folic acid.

The values for phospholipide obtained in these experiments agree closely with those found in the literature (5). Thus it would appear that glycine fed at a level of 10 per cent and especially that glycine plus ribonucleic acid produce an abnormal distribution of liver fatty acids, with a shift in the direction of increased neutral fat.

The results obtained in the present experiments indicate that in the absence of a sufficient quantity of folic acid in the diet there is a shift in the distribution of the liver fatty acids, with a relative increase in the quantity of neutral fat, even though the choline intake is presumably adequate. Folic acid then would seem to act as a lipotropic agent in that it tends to maintain a normal distribution of fatty acids.

Folic acid, when added to the diets, could act in any of several ways. It has been shown (6) that folic acid increases the rate of breakdown of glycine and its transformation to serine. If, in the absence of folic acid, glycine metabolism requires the excessive utilization of "labile" methyl groups, it is possible that folic acid reduces the drain on "methyl" groups by this mechanism and thus brings about a decrease in neutral fat. Folic acid has also been implicated in the metabolism of "formate" (7, 8), and, since "formate" can apparently serve as a precursor of "methyl" groups (9), folic acid might affect the supply of choline and other "methyl" donors by accelerating "methyl" neogenesis. Other explanations can also be given, but present knowledge does not permit a choice between the various possible mechanisms.

Dinning *et al.* (10) have recently found that the livers of rats fed a diet containing 1 per cent pyridine, supplemented with folic acid and vitamin B₁₂, showed evidence of fatty infiltration. The livers of rats receiving the unsupplemented pyridine diet appeared normal. It would thus appear that folic acid can exert either an antilipotropic or a lipotropic action, depending upon the experimental conditions.

SUMMARY

In rats maintained on various purified diets containing 18 per cent casein and 12 per cent fat the presence of folic acid in the diets has been shown to prevent an elevated neutral liver fat. This effect was noted not only in animals receiving the purified diet, but also in animals receiving similar diets containing 10 per cent glycine or 10 per cent glycine and 4 per cent ribonucleic acid.

Cholesterol, phospholipide, and total fatty acid determinations were made on the livers of these animals. The only significant alteration (from controls) in the cholesterol content of the livers was found when both glycine and ribonucleic acid were added to the diet. In this instance the cholesterol level was significantly elevated and this elevation was prevented by the addition of folic acid to the diet.

There appeared to be a slight reduction (from controls) in the phospholipide content of the livers of rats receiving the diet containing 10 per cent glycine. This reduction was prevented by the feeding of folic acid.

When ribonucleic acid was added to the diets containing a high level of glycine, there was a statistically significant elevation in the cholesterol and neutral fat content of the livers but no significant effect on the phospholipide content.

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ABSORPTION SPECTRA OF PROTEINS AND AMINO ACIDS AFTER ULTRAVIOLET IRRADIATION*

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Irradiation of proteins with ultraviolet light alters many of their physical and chemical properties (16) including the ultraviolet absorption spectrum. The present work is an attempt to explain the changes in this spectrum in chemical terms.

Methods

Egg albumin was purified according to the method of Kekwick and Cannan (9) except that it was lyophilized after the third recrystallization. The lyophilized preparation, which contained 14.3 per cent nitrogen, was dissolved in 0.05 M phosphate buffer, pH 7.3, to give a 1 per cent solution. The substances tested, in concentrations of 0.001 to 0.01 M, were dissolved in the buffer. The solutions were irradiated for 24 hours, with constant stirring, with a General Electric 30 watt mercury resonance lamp which has predominant radiation at 2537 Å. The dosage, as determined by a Hanovia ultraviolet meter, was approximately 1.7×10^7 ergs per sq. mm.

Absorption spectra were measured in the Beckman quartz spectrophotometer. The results are expressed as extinction coefficients ($(\log I_0/I)/cd$) where $\log I_0/I$ is the optical density, c is the concentration in gm. per 100 ml. for E or moles per liter for ϵ , and d is the thickness of the cell in cm.

RESULTS AND DISCUSSION

The absorption spectrum of egg albumin before and also after various periods of irradiation is shown in Fig. 1. The observations are similar to those of previous authors (1, 12, 18, 21). It should be noted that the extinction coefficient increases over the whole range of wave-lengths, but especially in the region of the original minimum around 2500 Å and also above 3000 Å where the unirradiated protein is almost non-absorbing.

Since Lerner and Barnum (11) have shown that the absorption of ultraviolet light by protein can be accounted for quantitatively in terms of the

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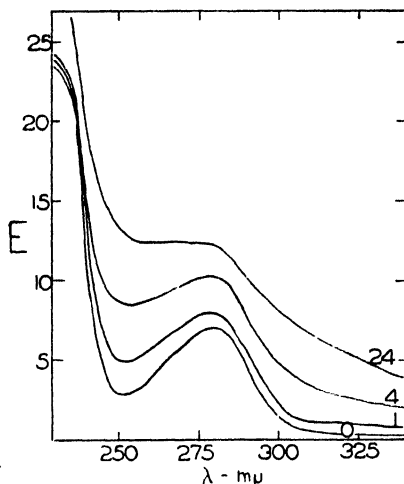


FIG. 1. The effect of ultraviolet light on the absorption spectrum of egg albumin. 1 per cent solutions in phosphate buffer, pH 7.3, were irradiated. The absorption curves were determined on 0.1 per cent solutions. The time of irradiation, in hours, is shown on each curve.

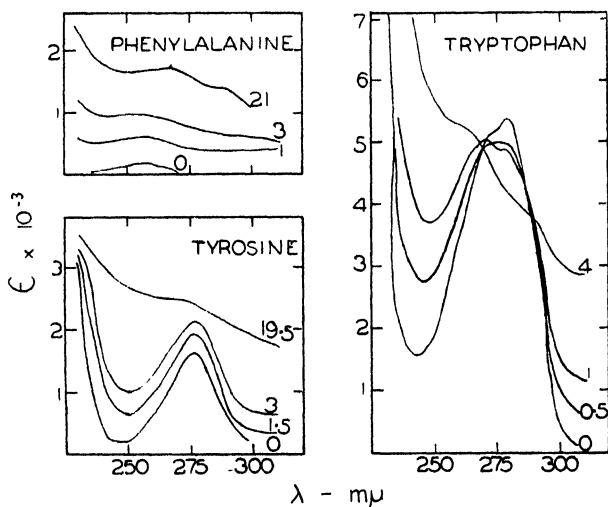


FIG. 2. The effects of ultraviolet light on the absorption spectra of tryptophan, tyrosine, and phenylalanine irradiated in phosphate buffer, pH 7.3. Tryptophan, irradiated as 0.001 M, absorption spectrum for 0.00025 M; tyrosine, irradiated as 0.0022 M, absorption spectrum for 0.002 M; phenylalanine, irradiated as 0.0025 M, absorption spectrum for 0.0017 M. The time of irradiation in hours is shown on each curve.

phenylalanine, tyrosine, and tryptophan which it contains, the next step was to expose these amino acids separately to ultraviolet light. The results of such experiments are shown in Fig. 2. Qualitatively, the changes in the spectra resemble those found with protein, an increase near 2800 Å after relatively short irradiation and a large increase at the original minima.

TABLE I

The molecular extinction coefficients of aromatic compounds at their maxima near 2800 Å. Many of the data have been converted from the units used in the original to those used here.

Compound	R group	$\lambda_{\text{max.}}$	ϵ	Solvent	Bibliographic reference No.
		λ			
Alanine	Alanyl	No absorption		HCl	(5), (13)
Benzene		2540	204	Dilute methanol	(3)
		2545	208	95% ethanol	(2)
		2540	162	Water	(10)
Toluene	CH ₃	2620	190	95% ethanol	(2)
		2610	225	Dilute methanol	(3)
Phenylalanine	Alanyl	2600	182	0.01 N HCl	(11)
		2570	195	0.1 N HCl	(13)
		2600	150	Buffer, pH 7.3	This paper
Phenol	OH	2695	1820	Water	(22)
		2700	1450	Dilute methanol	(3)
		2700	1440	0.001 N HCl	(10)
		2700	1430	Buffer, pH 7.3	This paper
<i>p</i> -Cresol	OH, CH ₃	2800	1850	95% ethanol	(8)
Tyrosine	OH, alanyl	2750	1300	0.01 N HCl	(11)
		2750	1620	Buffer, pH 7.3	This paper
Catechol	OH, OH	2755	2500	Water	(22)
		2750	2240	Buffer, pH 7.3	This paper
Dopa	OH, OH, alanyl	2800	2600	0.01 N HCl	(11)
		2800	2700	Water	(15)
Pyrogallol	OH, OH, OH	2650	830	Hexane	(7)
		2700	700	Buffer, pH 7.3	This paper
Indole		2650	6290	Hexane	(17)
Tryptophan	Alanyl	2760	5900	0.01 N HCl	(11)
		2800	5190	Water	(4)
		2770	5350	Buffer, pH 7.3	This paper

Although the greatest absorption prior to irradiation is found for tryptophan, by far the largest percentage increase in the extinction coefficient takes place with phenylalanine (the maximum at 2550 Å, increasing from 150 to 1750).

The rôle of the side chains of phenylalanine and tyrosine was then in-

vestigated by irradiating their analogues, benzene and phenol. The absorption spectra of these compounds closely resembled those of the corresponding amino acids, both before and after irradiation. Therefore any explanation of the nature of the chemical changes cannot ascribe an important rôle to the side chains or to the linkage of the amino acids in the protein.

The relative importance of the aromatic nucleus and of the side chains for the absorption of ultraviolet light is also demonstrated in Table I. This has been compiled from the literature and shows the magnitude of the absorption at the maximum in a number of phenolic compounds, including some amino acids. It is immediately apparent that the important

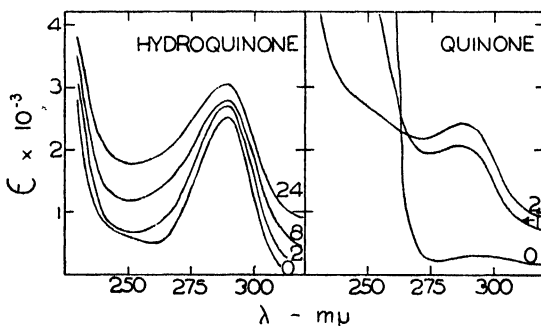


Fig. 3. The effects of ultraviolet light on the absorption spectra of quinone and hydroquinone irradiated in phosphate buffer, pH 7.3. Approximately 0.01 M solutions were used for irradiation and 0.001 M for the determinations of the extinction coefficients. The time of irradiation, in hours, is shown on each curve.

factor is the number of hydroxyl groups attached to the benzene ring. The molecular extinction coefficient varies from about 150 to 225 for compounds lacking hydroxyls, from 1300 to 1850 for those with one hydroxyl, and from 2250 to 2700 for those with two. However, the absorption at the maximum decreases again in the triphenol, pyrogallol.

Several lines of evidence suggest that ultraviolet light might cause an oxidation of some component of the protein. Harris (6) showed that aromatic amino acids and proteins containing these amino acids take up oxygen when they are irradiated. Also, the alterations of the absorption spectra as a result of exposure to ultraviolet light are similar to those found when proteins or phenols are oxidized in the presence of tyrosinase (14, 15, 19, 23), although phenylalanine, which is not attacked by tyrosinase, is altered by exposure to ultraviolet light. Finally it was found in preliminary experiments that ozone would bring about changes similar to those produced by ultraviolet light.

The first step in an oxidation would probably be the replacement of

some of the hydrogens on the benzene ring by hydroxyl groups. Therefore the formation of phenolic compounds from phenylalanine was tested with the Folin-Ciocalteu reagent ((20) p. 351) and the diazotized sulfanilic acid reagent ((20) p. 357). Neither reagent is very specific, but each indicated the formation of phenolic groups in up to 16 per cent of the phenylalanine molecules at one time. With protein, the sulfanilic acid reagent showed an increase in phenolic residues from the control value of 10 m.eq. to 37 m.eq. per 100 gm. after irradiation.

The data on absorption spectra are in accord with the postulate of oxidation. The formation of diphenols would lead to increased absorption at the maximum as already noted. Once a diphenol is formed, it would be oxidized to the corresponding quinone which in turn would be rapidly oxidized, even without ultraviolet light. The spectra of a diphenol, hydroquinone, and of the corresponding quinone, *p*-quinone, are shown in Fig. 3. Before irradiation, the spectrum of quinone differs markedly from that of the hydroquinone, but, after only 4 hours irradiation, it is similar to that of the diphenol after 24 hours irradiation. Therefore the absorption spectra indicate that a transitory quinone stage is not an unreasonable postulate.

SUMMARY

When protein is irradiated by ultraviolet light, qualitative and quantitative changes take place in its extinction coefficients at wave-lengths above 2400 Å. These effects can be interpreted as due to modifications of the aromatic portions of phenylalanine, tyrosine, and tryptophan, since similar changes occur in their analogues lacking alanyl side chains. Several lines of evidence indicate that the alterations are probably the result of oxidations.

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TESTS FOR HYDROLYSIS OF PEPTIDE BONDS BY ULTRAVIOLET LIGHT*

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It has been postulated that one of the primary actions of ultraviolet light on proteins is to hydrolyze the peptide bonds adjacent to the ultraviolet chromophores tyrosine, tryptophan, and phenylalanine (2, 3, 8, 10, 11). Since all the evidence is indirect, the present investigation was designed to test this hypothesis more adequately by performing chemical analyses for amino and carboxyl groups after doses of ultraviolet light sufficient to produce many physical changes in the protein.

Methods

The preparation of egg albumin, its irradiation, and the determination of its absorption spectrum were carried out as in previous work (7). Amino nitrogen was determined by the method of Van Slyke as modified by Grant (6), at a reaction time of 20 minutes. This method was rather unsatisfactory because of the precipitation of the protein. Basic groups were determined by the method of Fraenkel-Conrat and Cooper (5) with the dye orange G. The Sørensen formol titration as modified by Northrop (9) was used to determine the number of acidic groups.

Experimental Observations

The results of determinations of the number of m.eq. of amino groups, of basic groups, and of acidic groups per 100 gm. of protein are given in Table I. All the changes on irradiation are very small and none is significant at the 2 per cent level when tested statistically with the *t* test (4), although the increase in acidic groups is significant at the 5 per cent level.

In contrast, the absorption of ultraviolet light by the protein does increase considerably throughout the range of wave-lengths studied (2250 to 4000 Å), thus indicating that some chemical modification has taken place (7).

In similar experiments with a peptone, Bacto-tryptone, there was an

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increase in the extinction coefficient but no significant increase in the number of amino or acidic groups.

TABLE I

The concentrations in egg albumin of amino nitrogen, of basic groups, and of acidic groups and the changes produced by 24 hours of ultraviolet light irradiation. The values are m.eq. per 100 gm. of egg albumin.

Experiment No.	Amino N		Basic groups		Acidic groups	
	Control	Change	Control	Change	Control	Change
1	28.5	+1	98	-3.5		
2	37	+5				
3	26.5	+5	110	-6		
4	43	-4	92	+15		
5	43.5	-6.5	82	-11		
6	49.5	-9.5				
7			80	-1.5		
8			77	+5.5	66	+1
9					89	+5.5
10					59	+17
11					82	+14
12					80	+15.5
Mean ..	38	-1.5	90	-0.3	75	+10.6
S.d. ...	9		13		12	
S.d.m.		2.5		3.8		3.1

DISCUSSION

The only peptide bonds which are expected to be hydrolyzed by ultraviolet light are those adjacent to the chromophores tryptophan, tyrosine, and phenylalanine (3, 8). If these amino acids are freed completely from the protein, then two additional amino and carboxyl groups should be formed for each chromophore irradiated. Calculations based on the data of Block and Bolling (1), adjusted to the nitrogen content of the protein used, give for 100 gm. of egg albumin a total of 59 m.eq. of chromophore groups (21 from tyrosine, 6 from tryptophan, and 32 from phenylalanine). Thus if complete liberation occurs, it may be predicted that there will be 118 m.eq. of additional amino and carboxyl groups for each 100 gm. of protein hydrolyzed by irradiation.

Since the *E* values indicate over 99 per cent absorption of the incident light, approximately 250 quanta are absorbed for each chromophore if an average wave-length of 2537 Å is taken. Even though the increase in acidic groups is significant at the 5 per cent level, the quantum yield for the formation of these is only 7×10^{-4} .

It is obvious from the results that, if there is any formation of amino or acidic groups in addition to those present at the start, this is compensated for by the destruction of an approximately equal number of these groups but never of more than are formed. This would mean that newly formed groups are subject to some reaction that the ones originally present do not undergo, an improbable hypothesis.

Recent work (7) indicates that the small number of acidic groups formed might be transient phenolic groups, rather than carboxylic acids. This would explain the observation that no concurrent increase in the number of amino groups is observed, as would be expected if the peptide bond were hydrolyzed.

SUMMARY

1. The prediction that peptide bonds which are adjacent to aromatic residues in proteins can be hydrolyzed by ultraviolet light was tested by chemical analyses for the formation of amino and acidic groups on irradiation.

2. After extensive irradiation of egg albumin, a small but significant increase in the number of acidic groups was found. No significant change was found in the number of amino groups or of basic groups, although the methods used for all three analyses were sensitive enough to determine changes of less than one-tenth of the maximum predicted.

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EFFECT OF NIACIN PRECURSORS ON GROWTH OF RATS FED TRYPTOPHAN-LOW RATIONS*

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Previous work has shown that when purified rations containing 9 per cent casein are supplemented with certain proteins, acid-hydrolyzed proteins or single amino acids (threonine and phenylalanine), a reduction in the growth of rats results, which can be counteracted by the addition of niacin or tryptophan (1). Since the growth inhibition was counteracted by the addition of either niacin or tryptophan, it was decided that these rations could be used for testing compounds believed to be precursors of niacin.

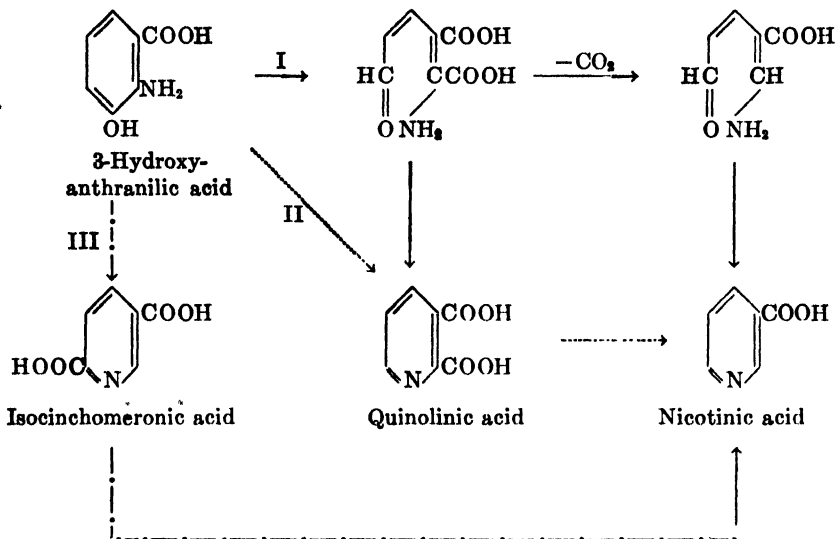
Recently several groups of workers have proposed schemes showing the intermediate compounds involved in the synthesis of niacin from tryptophan. On the basis of experiments with *Bacillus coli*, Ellinger and Abdel Kader (2) have suggested that ornithine is an intermediate in the synthesis of niacin from the amino acids, glutamic acid and arginine, and that nicotinamide rather than the acid is the substance synthesized from tryptophan in the intestinal tract. In addition they found that the formation of nicotinamide from ornithine could be accelerated by the addition of either choline or methionine, both of which are methyl donors. Work with mutant strains of *Neurospora* (3, 4) has led to several proposed schemes for the synthesis of niacin with anthranilic acid as the starting material. Most workers agree on the various steps of the synthesis up to the conversion of 3-hydroxyanthranilic acid. At this point three possible pathways may be followed.

Bonner and Yanofsky (5) have suggested that quinolinic acid is not an intermediate in the step from 3-hydroxyanthranilic acid to nicotinic acid. They propose that the 3-hydroxyanthranilic acid splits at the 3,4 position with decarboxylation and then ring closure to form nicotinic acid. Hen-

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dereson (6) has suggested that quinolinic acid is an intermediate, and he has shown that this compound accumulates when 3-hydroxyanthranilic



acid is incubated with liver homogenates (7). Another possible intermediate is isocinchomeronic acid, which would be formed if 3-hydroxyanthranilic acid were split at the 2,3 position. Experiments were conducted to test the niacin activity of some of these compounds with rations containing threonine, phenylalanine, or gelatin for the growth inhibition.

EXPERIMENTAL

The procedures used were similar to those employed in previous studies (8). Male Sprague-Dawley rats, 3 weeks of age, kept in individual cages, were fed *ad libitum* over a 5 week period. Each group contained three animals and in most cases a second group of rats was used. The various compounds thought to be involved in the synthesis of niacin were supplemented in rations containing added L-cystine and DL-threonine, DL-phenylalanine, or gelatin.

After the initial feeding experiments, several of the compounds were injected into the animals intraperitoneally. All the animals in these experiments were fed rations containing 0.156 per cent DL-threonine and were injected three times daily with 0.33 ml. of the various solutions. The compounds were dissolved in saline to prevent disturbance of the water balance of the animal.

In some of the experiments feces were collected during the 4th week. The feces were collected under alcohol, dried *in vacuo*, and samples weigh-

ing 0.2 gm. were autoclaved at 15 pounds pressure for 3.5 hours after the addition of 0.5 ml. of glacial acetic acid. After neutralizing, homogenizing, and diluting, the samples were assayed for "total" niacin, according to the method of Krehl *et al.* (9). Another portion of feces weighing 0.2 gm. was homogenized, neutralized, and assayed directly for "free" niacin. The difference between the free and total niacin values was considered as bound niacin obtained from the breakdown of bacterial cells.

After completion of the above experiments, groups of animals were fed the 9 per cent casein basal ration with added tryptophan, anthranilic acid, *p*-aminosalicylic acid, *p*-aminobenzoic acid, and quinolinic acid. During the 4th week of this experiment feces were collected from all the animals and treated as in the preceding experiment.

RESULTS AND DISCUSSION

The activity of the various compounds added to the 9 per cent casein rations containing additional threonine or phenylalanine is shown in Table I. The results are based on several separate experiments and in each case sucrose was used as the carbohydrate. Growth rates are presented as percentage of the growth of the negative control groups. If the added niacin precursors increased the growth of the rats to 90 per cent of the negative controls, they were considered active in the synthesis of niacin. Animals fed the 0.156 per cent DL-threonine ration grew at a reduced rate of approximately 28 per cent of the negative controls, and this growth reduction was overcome by the addition of 50 mg. of tryptophan per 100 gm. of ration. Anthranilic acid was added alone and in combination with serine or ornithine in order to provide the bacteria in the tract with material from which possibly to synthesize 3-hydroxyanthranilic acid by way of kynurenine or a structurally similar substance. The addition of these compounds as well as ornithine or indole and serine alone or in combination was ineffective in overcoming the DL-threonine inhibition. The inactivity of these precursors at the levels employed may have been due to the destruction of the compounds by certain forms of bacteria in the intestinal tract or to the toxicity of the compounds used. These compounds were also injected at levels equivalent to those fed. The injection of anthranilic acid plus ornithine was the only combination that showed slight activity.

When these compounds were fed with the phenylalanine rations, 33.6 mg. of anthranilic acid per 100 gm. of ration were only slightly active against the phenylalanine inhibition; however, when the level of anthranilic acid was doubled (67 mg.), a corresponding increase in activity occurred. The combination of 33.6 mg. of anthranilic acid and 32 to 65 mg. of ornithine per 100 gm. of the diet counteracted the phenylalanine in-

hibition. Ornithine alone was not active. From the results obtained it is evident that ornithine plus anthranilic acid or anthranilic acid alone

TABLE I
Effect of Niacin Precursors on Threonine or Phenylalanine Inhibition

Sucrose ration	Supplement	Gain, per cent of negative controls
Fed per 100 gm. ration		
0.156% DL-thre- onine	None	28
	50 mg. DL-tryptophan	125
	33.6 mg. anthranilic acid	58
	33.6 " " " + 25.8 mg. serine	51
	33.6 " " " + 32.4 " ornithine	58
	33.6 " " " + 64.8 " "	47
	28.7 " indole + 25.8 mg. serine	48
	57.4 " " + 51.6 " "	32
	32.4 " ornithine	45
	64.8 " "	50
	156 " serine	18
	30 " indole	25
	30 " " + 156 mg. serine	11
Injected per rat per day		
	3.4 mg. anthranilic acid	59
	3.4 " " " + 2.6 mg. serine	58
	3.4 " " " + 3.4 " ornithine	74
	6.5 " ornithine	43
	5.0 " isocinchomeric acid	49
Fed per 100 gm. ration		
0.208% DL-phen- ylalanine	None	23
	33.6 mg. anthranilic acid	77
	33.6 " " " + 25.6 mg. serine	41
	33.6 " " " + 32.4 " ornithine	96
	33.6 " " " + 64.8 " "	83
	28.7 " indole + 25.8 mg. serine	57
	57.4 " " + 51.6 " "	15
	32.4 " ornithine	33
	64.8 " "	64
	67 mg. anthranilic acid	148

either stimulated the synthesis of niacin or was actually utilized as part of the niacin molecule in the synthesis of the vitamin.

Further studies were conducted with the gelatin and threonine rations

to determine the effect of these compounds on the synthesis of niacin in the intestinal tract. It was thought that these compounds (including gelatin and threonine) may stimulate the synthesis of bound (unavailable) niacin in the intestinal tract as well as the various other tryptophan metabolites such as indoleacetic acid, indoleethylamine, and indolepyruvic acid, and consequently waste tryptophan, which is already limiting in the ration. It has been shown repeatedly that 6 per cent gelatin will reduce the growth of rats on a 9 per cent ration whether dextrin or sucrose is used as the carbohydrate, while the other growth-inhibiting materials reduce growth only when sucrose is the carbohydrate (1). Therefore the subsequent experiments were designed to test the effect of the various precursors and inhibitors on fecal niacin excretion and rat growth when sucrose and dextrin rations were used.

Considering the effect of the various added components (Table II), it may be seen that either 1.5 mg. of niacin or 50 mg. of DL-tryptophan per 100 gm. of the diet counteracted the gelatin inhibition completely with both the sucrose and dextrin rations. It is noteworthy that the growth of the animals fed the sucrose diet was almost double that of the negative controls. When anthranilic acid was added to the gelatin rations at 1 (33.5 mg.), 2, or 4 times the equimolar quantity of 50 mg. of tryptophan, no response was obtained. It is interesting that the equimolar amount of quinolinic acid (41 mg. per 100 gm. of ration), though it gave a lower response than the 50 mg. of tryptophan, brought the growth of the animals up to that of the controls. Lower levels were progressively less active. Isocinchomeronic acid (41 mg.) was inactive when fed at a level equivalent to 50 mg. of tryptophan. The combination of 32.4 mg. of ornithine and 67 mg. of anthranilic acid gave some response with the dextrin ration but relatively no response with the sucrose diet. It is evident that these two components increased the growth of the animals by an acceleration of niacin synthesis in the intestinal tract. It has been repeatedly shown that dextrin is a better medium for niacin synthesis than sucrose and, with these two precursors present, apparently much more synthesis occurred.

The data (Table II) obtained when the various niacin precursors were added to the threonine rations with sucrose as the main carbohydrate source were similar and showed the same trends. As with the gelatin ration, the 41 mg. level of quinolinic acid brought the growth of the animals up to or slightly higher than the controls. On the other hand, anthranilic acid, isocinchomeronic acid, and the combination of ornithine and anthranilic acid as well as quinolinic acid improved the growth of animals fed the dextrin ration containing threonine and phenylalanine. Tryptophan, however, was not effective. These results also indicate varia-

TABLE II

Effect of Precursors on Growth and Niacin Excretion

Ration	Supplement, per 100 gm. ration	Sucrose ration			Dextrin ration		
		Gain, per cent of negative controls	Fecal niacin, γ per day per 100 gm.		Gain, per cent of negative controls	Fecal niacin, γ per day per 100 gm.	
			Free	Total		Free	Total
Basal	None	100	5	10	100	6	29
	1.5 mg. niacin	157	5	8	109	32	32
	50 " tryptophan	112	11	15	119	46	52
6% gelatin	None	23	17	20	33	22	22
	1.5 mg. niacin	179	10	14	98	58	70
	50 " tryptophan	181	11	16	142	45	42
	34 " anthranilic acid	12					
	67 " " "	26	12	14	52	35	28
	134 " " "	19	17	93	52		
	41 " quinolinic acid	111	18	95	83	92	730
	20 " " "	63					
	2 " " "	32					
	41 " isocinchomeronic acid	21	22	26	39		
	32.4 mg. ornithine + 67 mg. anthranilic acid	39	15	17	80		
0.156% DL-threonine	None	32					
	1.5 mg. niacin	188					
	50 " DL-tryptophan	147					
	34 " anthranilic acid	29					
	67 " " "	34	11	14			
	134 " " "	37					
	41 " quinolinic acid	126	13	378			
	20 " " "	88					
	2 " " "	54					
	41 " isocinchomeronic acid	38					
	32.4 mg. ornithine + 67 mg. anthranilic acid	31					
0.18% DL-threonine + 0.24% DL-phenylalanine*	None				84	37	36
	1.5 mg. niacin				140		
	50 " DL-tryptophan				85	46	48
	67 " anthranilic acid				120	30	30
	41 " quinolinic acid				134	20	2250
	41 " isocinchomeronic acid				101		
	32.4 mg. ornithine + 67 mg. anthranilic acid				120		

* These amounts of threonine and phenylalanine were calculated to equal the levels of the amino acids contributed by 6 per cent gelatin.

tions in the synthesis of niacin by intestinal bacteria when the dietary carbohydrate differs.

The activity of the 41 mg. level of quinolinic acid against the gelatin and threonine inhibition could be considered as evidence that it is the direct precursor of niacin in the niacin synthesis scheme. However, it is evident that this level of quinolinic acid is not as active as the equimolar quantity of tryptophan. This lowered activity could be due to the toxicity of such a massive quantity of the compound. On the other hand, if quinolinic acid is the direct precursor of niacin, the 2 mg. level should give the same response as 1.5 mg. of niacin, since metabolism studies in this laboratory showed that quinolinic acid was quite readily absorbed (88 per cent from the intestinal tract (10)). Nevertheless, there is still the possibility that the intestinal bacteria utilized or destroyed the small quantity of the compound added to the ration.

The fecal niacin data from the animals fed the sucrose rations (Table II) showed that the niacin added to the ration was readily absorbed by the animal. When tryptophan as well as the other materials were added, free niacin excretion increased significantly with the dextrin ration and slightly with the sucrose diet. This could be the result of an increased synthesis of coenzymes in the intestinal tract. Coenzyme I as well as nicotinamide and free niacin is equally active in the *Lactobacillus arabinosus* assay (11). Since niacin formed in the intestinal tract is easily absorbed, the increases in the free niacin values may have been due to a factor which was not absorbed from the tract (coenzymes), but was active in the niacin assay. The greatest increase in free niacin (15-fold) was obtained with quinolinic acid added to the gelatin dextrin ration. The addition of quinolinic acid to the sucrose ration increased free niacin in the feces only 4-fold.

The total (free and bound) niacin values (Table II) show that no large quantities of bound niacin were synthesized in the intestinal tracts of animals with the exception of those fed rations containing quinolinic acid. With the addition of 41 mg. of quinolinic acid to a dextrin diet containing 6 per cent gelatin the increase in total niacin excreted by the animals was 4-fold higher than when the animals were fed a sucrose ration containing the same materials. This difference could have been the result of a greater stimulation of synthesis of bound niacin with the dextrin diet or it could have been a greater retention of quinolinic acid in the intestinal tract.

Further consideration of the fecal niacin data (Table II) shows that the addition of the various precursors to the gelatin or threonine rations containing sucrose gave slight increases in the total fecal niacin. It is interesting that gelatin alone increased the value of free and total niacin, but not to the extent that the increased synthesis of the vitamin could be

considered to have critically lowered the tryptophan level of the ration. Also significant in this experiment are the total niacin values obtained when the two levels of anthranilic acid were added to the sucrose ration containing 6 per cent gelatin. The low level (67 mg. per 100 gm. of ration) of anthranilic acid neither stimulated the niacin synthesis nor overcame the gelatin growth inhibition, while the higher level (134 mg.), though it had no stimulatory effect on growth, caused a 9-fold increase in total niacin excreted. It is evident that this compound is involved in the synthesis, or at least can stimulate the synthesis, of niacin in the intestinal tract.

From the data presented it is evident that the type of carbohydrate and the kind and amount of possible niacin precursor present in a ration all affect the response of the bacterial population of the intestinal tract.

TABLE III

Effect of Various Compounds on Growth and Niacin Excretion with 9 Per Cent Casein Basal Ration

Supplement, per 100 gm. ration	Gain, per cent of negative controls	Total fecal niacin, γ per day per 100 gm.
None		11.2
50 mg. tryptophan	125	8.7
67 " anthranilic acid	157	10.1
134 " " "	120	10.5
75 " <i>p</i> -aminosalicylic acid	129	7.7
150 " " "	93	14.1
67 " <i>p</i> -aminobenzoic acid	100	10.8
134 " " "	122	7.6
41 " quinolinic acid	131	277.0

Depending on the level of the compound fed, there apparently is a selective inhibition or stimulation of the various bacteria involved in the synthesis of free or bound niacin.

It is possible that any compound with an amine group in a position similar to anthranilic acid could stimulate the synthesis of niacin. To test this, several compounds similar in structure to anthranilic acid were fed in the 9 per cent casein basal sucrose ration without other supplement. The growth responses and fecal niacin excretions obtained upon the addition of tryptophan, quinolinic acid, anthranilic acid, *p*-aminosalicylic acid, or *p*-aminobenzoic acid are presented in Table III. All the compounds except *p*-aminobenzoic acid at 67 mg. and *p*-aminosalicylic acid at 150 mg. per 100 gm. of ration had a stimulatory effect on the growth of the rats, while none of them had any significant effect on the niacin level of the feces. The two anthranilic acid levels (67 mg. and 134 mg. per 100

gm. of ration) showed the same effects on growth in this experiment as when they were used in a gelatin ration (Table II). The growth of animals receiving the higher level of anthranilic acid was lower in both experiments. The total niacin excreted by the animals supplemented with 134 mg. of anthranilic acid on a 6 per cent gelatin ration (Table II) was greater than the quantity excreted by the animals fed the 134 mg. of anthranilic acid alone (Table III). This is additional evidence that the form of niacin synthesized is dependent on a number of factors. The amount of niacin in the feces cannot be correlated directly with the growth response.

SUMMARY

The effect of postulated niacin precursors on the growth of animals fed a 9 per cent casein ration supplemented with 6 per cent gelatin, 0.156 per cent DL-threonine, or 0.2 per cent DL-phenylalanine, was determined.

The combination of 33.6 mg. of anthranilic acid and 32.4 mg. of ornithine per 100 gm. of diet completely overcame the phenylalanine inhibition. Isocinchomeronic acid, anthranilic acid, and ornithine combined, and various levels of anthranilic acid, were all inactive in both the gelatin and threonine rations. When dextrin was used as the carbohydrate source, anthranilic acid and ornithine in combination produced a growth response. 41 mg. of quinolinic acid per 100 gm. of ration gave good growth response on both the gelatin and threonine diets.

The form of nicotinic acid synthesized in the intestinal tract (free or bound) is determined by the type of ration fed and the kind of niacin precursor supplied. Niacin synthesis in the tract cannot be correlated directly with the growth response.

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THE RESOLUTION OF ISOEQUILIN A AND THE IDENTIFICATION OF COMPOUND 3

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In a previous study (1) it was noted that equilin developed a blue color when heated with phenolsulfonic acid in the presence of ferric salts (iron-Kober reagent). Although estrone and equilenin produced only negligible pink hues under the conditions of the test,¹ the reaction could not be utilized in the assay of urine extracts for equilin. With all such extracts equilin recoveries based upon chromogenic activity in the iron-Kober test were significantly higher than the results obtained by the use of the infra-red spectrophotometric method (2). The colorimetric procedure subsequently devised, including the reaction of esterified equilin with dibromoquinonechloroimide reagent (1), corroborated the infra-red findings, and indicated that unidentified phenolic ketosteroids related to equilin occur in the estrogenic hormone fraction obtained from equine pregnancy urine.

In an attempt to ascertain the nature of these substances, several derivatives of equilin were studied. Isoequilin A was prepared according to the procedure of Hirschmann and Wintersteiner (3), and it also produced a blue color when heated with iron-Kober reagent. However, the intensities of the colors produced by different samples of isoequilin A varied. Discrepancies were also noted in the ultraviolet absorption properties of the various samples, the wave-length of maximum absorption ranging from 267 to 270 $m\mu$, while the original investigators had reported a λ_{max} at 272 $m\mu$ for their isoequilin A. No estrogenic material with maximum absorption at any of these wave-lengths has been detected in pregnancy urine.

Hirschmann and Wintersteiner (4) have isolated a urinary diol isomeric with dihydroequilin, which they designated "Compound 3." Through the courtesy of Dr. D. Beall of Ayerst, McKenna and Harrison, Ltd., we

¹ 1 part of crystalline phenol is dissolved without cooling in 1.13 parts by weight of sulfuric acid containing 9 mg. of ferric ion per 100 ml. of acid. After 16 to 24 hours there are added to the product 9 times its volume of diluted sulfuric acid (1 + 1 by volume). Alcoholic solutions of estrogens (2 ml., containing 100 to 200 γ) are mixed with 5 ml. of the reagent, and heated at 75–80° for 1 hour. Equilin develops a deep blue color with λ_{max} at 625 $m\mu$.

were supplied with a large quantity of crude Compound 3. The purified substance (m.p. 233–234°; $[\alpha]_D^{20} = +130^\circ$ in ethanol), isolated by partition chromatography by the procedure of Haenni (5), exhibited a characteristic ultraviolet absorption spectrum with a major peak at 263.5 $m\mu$ ($\epsilon = 18,000$), a secondary peak at 300 $m\mu$, and minima at 289 and 235 $m\mu$ (Curve A, Fig. 1). The 17-ketosteroid corresponding to Compound 3 was prepared by subjecting the diol to an Oppenauer oxidation (6), and the ketone was isolated by means of Girard's Reagent T (7).

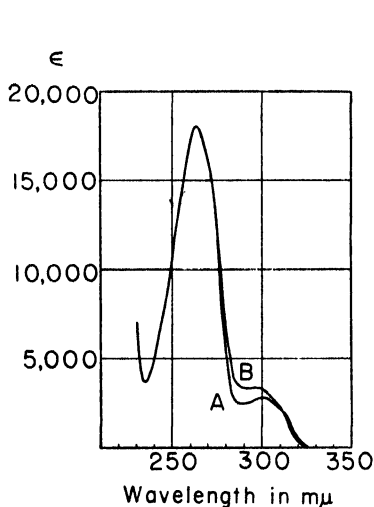


FIG. 1

FIG. 1. The ultraviolet absorption spectra of Compound 3 (Curve A) and of 9-dehydro-14-isoestrone (Curve B), in ethanol.

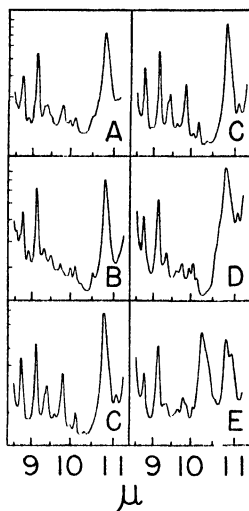


FIG. 2

FIG. 2. The infra-red absorption spectra of the benzenesulfonate esters of isoequilin A (Curve A), 8-dehydro-14-isoestrone (Curve B), 9-dehydro-14-isoestrone (Curve C), Compound 3 (Curve D), and β -9-dehydro-14-isoestradiol (Curve E). Equivalent of 4 mg. of estrogen per ml. of CS_2 .

Its ultraviolet absorption spectrum (Curve B) resembled that of Compound 3 closely. (The greater extinction values observed for the ketone between 280 and 310 $m\mu$ may be due in part to the contributory absorption of its carbonyl system.)

When treated with iron-Kober reagent, the new ketosteroid developed an intense blue color. Comparison of the infra-red absorption spectrum of its benzenesulfonate, prepared by the procedure of Carol, Molitor, and Haenni (2) (Curve C, Fig. 2), with that of isoequilin A benzenesulfonate (Curve A) revealed significant similarities between the two, and suggested that isoequilin A was probably a mixture consisting chiefly of

the new substance and another ketosteroid closely related to it. This supposition would explain the discordant data obtained for different preparations of isoequilin A as being due to variability in the proportion of these constituents. Hirschmann and Wintersteiner themselves (3) had expressed doubts concerning the homogeneity of their product, although it was unresolvable by either fractional crystallization or adsorption chromatography.

By repeated application of partition chromatography, with the use of 0.4 N sodium hydroxide solution adsorbed on Celite² as the immobile

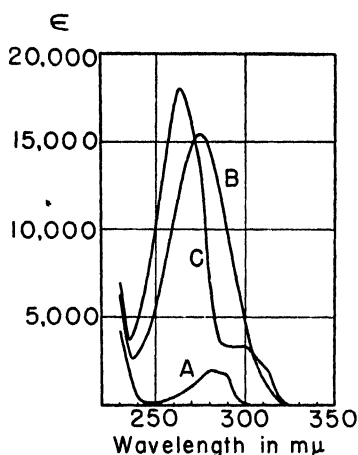


FIG. 3

Fig. 3. The ultraviolet absorption spectra of equilin (Curve A), 8-dehydro-14-isoestrone (Curve B), and 9-dehydro-14-isoestrone (Curve C), in ethanol.

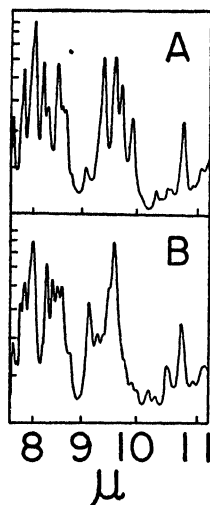


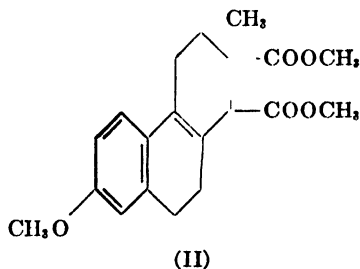
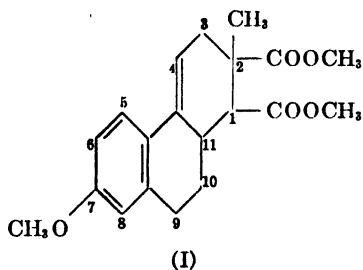
FIG. 4

Fig. 4. The infra-red absorption spectra of equilenin methyl ether (Curve A) and isoequilenin methyl ether (Curve B), 15 mg. per ml. of CS₂.

phase and benzene as the mobile solvent, we have been able to resolve isoequilin A into its component ketosteroids. One of these components, constituting 40 to 50 per cent of the crude reaction mixture, was identical with the ketosteroid prepared by the oxidation of Compound 3. Another, present to the extent of approximately 45 per cent, was characterized by an ultraviolet spectrum with maximum absorption at 275 mμ ($\epsilon = 15,900$) and a minimum at 237 mμ ($\epsilon = 2800$, Curve B, Fig. 3). In addition, small quantities of crude equilenin and unchanged equilin were obtained, together with colored substances adsorbed on the column and oils recovered from the forerun.

² Celite No. 545, manufactured by Johns-Manville.

Equilin was detected by means of dibromoquinonechloroimide reagent (1) after esterification of the residues in the first eluate fractions of the chromatogram. Equilenin was isolated by means of its picrate from the final portions of eluate received, and the phenolic group was methylated. The infra-red absorption spectrum of the equilenin methyl ether so obtained proved to be identical with the spectrum given by *d*-equilenin methyl ether, and markedly different from that of *d*-isoequilenin methyl ether (Curves A and B, Fig. 4). However, when each of the unidentified ketosteroids was heated with acetic-hydrochloric acid mixture, isoequilenin (VII) was recovered by chromatography. These findings indicate that the two major constituents of isoequilin A have the *cis* configuration in the C to D ring fusion, and that epimerization occurs at C₁₄ during the isomerization of equilin, as previously concluded by Hirschmann and Wintersteiner (3).



The high ultraviolet molecular extinction of the two unidentified ketosteroids indicated that both possessed a fourth double bond in conjugation with the benzenoid nucleus. Anner and Miescher (8) and Heer and Miescher (9) have synthesized hexahydrophenanthrene derivatives which yield ultraviolet absorption spectra almost identical with the curves obtained by us. The data reported by Heer and Miescher (9) for the dimethyl ester of 2-methyl-7-methoxy-1,2,3,9,10,11-hexahydrophenanthrene-1,2-dicarboxylic acid (I) include maxima at 264 $m\mu$ ($\epsilon = 20,900$) and 299 $m\mu$, and minima at 289 and 234 $m\mu$, while those for the dimethyl ester of 2-methyl-7-methoxy-1,2,3,4,9,10-hexahydrophenanthrene-1,2-dicarboxylic acid (II) yield a simpler curve with a maximum at 275 $m\mu$ ($\epsilon = 18,500$) and a minimum at 238 $m\mu$. (The methyl ethers of our ketosteroids, prepared by the action of methyl sulfate on their solutions in alkali, provided ultraviolet absorption spectra identical with those of the free phenols.) On the basis of the close agreement of our data with those reported by Heer and Miescher (9) for their synthetic compounds of known structure, we believe our ketosteroid with the absorption peak at 275 $m\mu$ to be 8-dehydro-14-isoestrone (IV), and that with the maxima at 263.5 and 299 $m\mu$ to be 9-dehydro-14-isoestrone (V).

8-Dehydro-14-isoestrone is easily soluble in ethanol and methanol, and crystallizes in shining white rhomboidal platelets (m.p. 232–233°; $[\alpha]_D^{20} = +136^\circ$ in dioxane and $+142^\circ$ in ethanol). These properties suggest the identity of 8-dehydro-14-isoestrone with hippulin (m.p. 233°; $[\alpha]_D^{18} = +128^\circ$ in dioxane), an isomer of equilin isolated by Girard and his co-workers (10) from equine pregnancy urine, but encountered by no other investigators since its discovery. However, our ketosteroid evinces only slight estrogenic activity,³ whereas hippulin was reported to possess a potency equal to that of equilin (10).

In the iron-Kober test 8-dehydro-14-isoestrone develops a blue color about one-third as intense as the color due to equilin, and its benzenesulfonate is inert toward dibromoquinonechloroimide (1), while the free phenol develops a negligible orange color with the reagent. The ketosteroid yields a crystalline methyl ether and acetate, and forms a water-soluble derivative with Girard's Reagent T (7), from which the original substance can be recovered, with slight decomposition. When heated with acetic-hydrochloric acid mixture, 8-dehydro-14-isoestrone is partially converted to 9-dehydro-14-isoestrone, indicating the mechanism for the formation of the latter substance in the isomerization of equilin.

9-Dehydro-14-isoestrone crystallizes from ethanol as columnar crystals (m.p. 254–255°; $[\alpha]_D^{20} = +293^\circ$ in dioxane and $+311^\circ$ in alcohol). It yields a crystalline acetate and methyl ether, and forms a water-soluble derivative with Girard's Reagent T from which the ketosteroid can be recovered quantitatively. When heated with iron-Kober reagent, it develops a blue color about equal in intensity to that due to equilin, and it is inert toward dibromoquinonechloroimide reagent after esterification. The estrogenic potency of 9-dehydro-14-isoestrone is about one-third the potency of estrone.³

By the use of lithium aluminum hydride, 17-ketosteroids are converted to alcohols of the β form⁴ exclusively (13, 14), and β -9-dehydro-14-isoestradiol has been prepared from 9-dehydro-14-isoestrone in this manner. Its ultraviolet absorption spectrum is identical with the spectrum shown for Compound 3 (Curve A, Fig. 1). The infra-red absorption spectrum of its dibenzenesulfonate (Curve E, Fig. 2) exhibits the properties which

³ We are indebted to Dr. J. M. Curtis and to Miss N. M. Hromadka of the Division of Pharmacology, Food and Drug Administration, for performing this bioassay.

⁴ Fieser and Fieser (11) have recently cited evidence that the active estrogenic diol occurring in human pregnancy urine, hitherto referred to in the literature as " α -estradiol," is actually estradiol-17 β (*cis*). The nomenclature employed in this report conforms to the newer usage. Thus, the more recently isolated diol derived from equilin (12) is here designated as " α -dihydroequilin," while diols obtained by the reduction of ketosteroids with lithium aluminum hydride are called " β -dihydro" compounds.

would be expected for the β -17-dihydro derivative of 9-dehydro-14-isoestrone (appearance of a new band at $10.10\ \mu$, in addition to the bands shown by the ketosteroid). The new diol crystallizes from 50 per cent ethanol as white clustered bipyramidal crystals (m.p. $182-184^\circ$; $[\alpha]_D^{20} = +135^\circ$ in ethanol).

The relationship of the infra-red absorption spectrum of 9-dehydro-14-isoestrone benzenesulfonate (Curve C, Fig. 2) to the spectrum of the dibenzenesulfonate of Compound 3 (Curve D, Fig. 2) is analogous to the relationship between the previously reported spectra for estrone (2) and estradiol-17 α (15), equilin (2) and α -dihydroequilin (12), and equilenin (2) and α -dihydroequilenin (15). Compound 3 is therefore designated as α -9-dehydro-14-isoestradiol (IX). This identification is confirmed by the fact that Compound 3 was recovered in 12 per cent yield when α -dihydroequilin (VIII) was heated with 0.5 N hydrochloric acid, an isomerization analogous to the conversion of equilin (III) to 9-dehydro-14-isoestrone (V) in acid media.

By the use of partition chromatography we have been able to isolate 9-dehydro-14-isoestrone from commercial samples of estrogenic materials. The presence of this substance explains the blue colors obtained in testing mixed estrogens with iron-Kober reagent, even when equilin is shown to be absent by analysis of the infra-red data and by a negative test with dibromoquinonechloroimide reagent after esterification. The quantity of 9-dehydro-14-isoestrone detected in urine extracts is increased with greater concentrations of acid and longer heating periods employed in the hydrolysis of the phenolic esters.

The occurrence of Compound 3 in urine extracts is even more significant. Grant and Beall (16) have demonstrated that samples with a high conjugated estrogenic diol content generate Compound 3 when hydrolyzed with acid but not when split with dioxane. Upon treatment with dioxane, the diol fractions obtained from samples rich in equilin yield mixtures with infra-red absorption properties characteristic of α -dihydroequilin.⁵ When such samples are hydrolyzed with acid, the liberated diols exhibit the ultraviolet and infra-red properties of Compound 3, but not those of α -dihydroequilin. These facts indicate that α -9-dehydro-14-isoestradiol (Compound 3) is derived from α -dihydroequilin as an artifact in the acid hydrolysis of the natural conjugated estrogens. 9-Dehydro-14-isoestrone is obtained in the same manner by the acid isomerization of equilin during the hydrolytic process.

EXPERIMENTAL

Purification of Compound 3—Crude Compound 3 (150 mg.), contaminated with α -dihydroequilenin, was dissolved in 20 ml. of benzene and

⁵ Carol, J., unpublished work.

chromatographed by the partition method of Haenni (5) on a column made up of 21 gm. of Celite and 20 ml. of 0.4 N sodium hydroxide in a 25×250 mm. tube. After discarding 200 ml. of benzene eluate, 50 ml. portions were collected. Those residues (from Fractions 8 to 14) showing an ultraviolet $\lambda_{\max}$. at $264 \text{ m}\mu$, and no evidence of α -dihydroequilenin, were combined. The combined residue was dissolved in 20 ml. of benzene and rechromatographed on a fresh column in a similar manner. All residues with $\lambda_{\max}$. at 263.5 and $300 \text{ m}\mu$ and $\lambda_{\min}$. at $289 \text{ m}\mu$ were combined and twice recrystallized from 80 per cent ethanol (m.p.⁶ $233\text{--}234^\circ$; $[\alpha]_D^{20} = +130^\circ$, 1 per cent in ethanol). The ultraviolet absorption spectrum is shown in Curve A, Fig. 1.

Oxidation of Compound 3—Compound 3 (25 mg.) was dissolved in 5 ml. of dry acetone and mixed with 10 ml. of dry benzene containing 1 gm. of aluminum *tert*-butoxide. The mixture was refluxed 8 hours, cooled, and mixed with 25 ml. of ether. The ethereal solution was extracted with dilute hydrochloric acid and water, and evaporated to dryness. Ketosteroids in the residue were isolated by means of Girard's Reagent T (7) and recrystallized twice from 50 per cent ethanol. Yield, 4.5 mg. of white columnar crystals melting at $245\text{--}246^\circ$ to a blue liquid. The ultraviolet absorption spectrum of the substance and the infra-red absorption spectrum of its benzenesulfonate proved to be identical with the corresponding curves given by 9-dehydro-14-isoestrone (Curve B, Fig. 1, and Curve C, Fig. 2). The chromatographic properties of the two samples were also the same.

Resolution of Isoequilin A—Equilin (500 mg., m.p. $237\text{--}239^\circ$; $[\alpha]_D^{20} = +325^\circ$, 0.5 per cent in ethanol) was isomerized by heating with 50 ml. of acetic-hydrochloric acid mixture, according to the procedure of Hirschmann and Wintersteiner (3). (The reaction proceeds as readily at 100° in 20 ml. of acetic acid plus 5 ml. of hydrochloric acid.) The cooled solution was diluted with 200 ml. of water and extracted with two 75 ml. portions of peroxide-free ether. After cautious washing with two 40 ml. portions of 10 per cent sodium carbonate solution and two 40 ml. portions of water, the ether extracts were combined and evaporated to dryness on the steam bath, with the aid of a current of air. The residue was dissolved in 50 ml. of hot benzene, and chromatographed on a column of 31 gm. of Celite and 30 ml. of 0.4 N sodium hydroxide. A forerun of 100 ml., containing 55 mg. of oils and unchanged equilin, was discarded,

⁶ All melting points reported were obtained on a Fisher-Johns melting point apparatus calibrated against standard corrected thermometers. Unless otherwise noted, samples were placed on the heating stage 10° below the melting point, and the temperature raised 1° per minute until all crystals liquefied.

and 20 ml. fractions were collected. Fractions 3 to 6 contained 124 mg. of material, with $\lambda_{\max}$. 274–276 $m\mu$ and $\epsilon = 10,000$ to 15,000 (chiefly 8-dehydro-14-isoestrone); Fractions 7 to 11 contained 130 mg. with $\lambda_{\max}$. 264–273 $m\mu$, $\epsilon = 15,500$ to 17,500; Fractions 12 to 19 contained 121 mg. of material with $\lambda_{\max}$. 264 $m\mu$, $\epsilon = 16,000$ to 18,000 (chiefly 9-dehydro-14-isoestrone); Fractions 20 to 30 contained 12 mg. of a material with an ultraviolet absorption spectrum resembling that of equilenin. The combined residues from Fractions 5 to 9, representing the zone of overlapping between 8-dehydro-14-isoestrone and 9-dehydro-14-isoestrone were dissolved in 20 ml. of hot benzene and rechromatographed. A third chromatogram, applied to the mixed material obtained from the second, left 21 mg. of material with $\lambda_{\max}$. 264–273 $m\mu$, which was discarded. The crude 8- and 9-dehydro-14-isoestrones isolated in these chromatograms (about 45 mg. in each case) were combined with the appropriate eluates previously recovered.

8-Dehydro-14-isoestrone—Crude 8-dehydro-14-isoestrone (170 mg.) was chromatographed on a column of 31 gm. of Celite and 30 ml. 0.4 N sodium hydroxide. The bulk of the material with $\lambda_{\max}$. 273–275 $m\mu$ and $\epsilon = 11,500$ to 16,000 was received in the second 125 ml. portion of eluate. This material was again chromatographed on a fresh column, and 120 mg. of material with $\lambda_{\max}$. 274–275 $m\mu$ and $\epsilon = 13,500$ to 16,000 were isolated. The slightly impure substance was recrystallized three times from methanol, yielding 84 mg. of shining white rhomboid platelets, $[\alpha]_D^{20} = +142^\circ$ (0.5 per cent in ethanol) and $+136^\circ$ (0.5 per cent in dioxane). The crystals acquired a purple coloration at 228° and melted to a blue liquid at 232 – 233° .

Analysis— $C_{18}H_{20}O_2$. Calculated. C 80.55, H 7.52
Found. " 80.41, " 7.38

500 γ of 8-dehydro-14-isoestrone benzenesulfonate, treated with dibromoquinonechloroimide reagent, yielded no more color than a reagent blank. When heated with iron-Kober reagent, 10 γ of the substance produced a blue color about one-third as intense as that due to an equal weight of equilin.

9-Dehydro-14-isoestrone—Crude 9-dehydro-14-isoestrone (165 mg.), obtained from isoequilin A, was dissolved in 40 ml. of hot benzene and chromatographed on a column of 21 gm. of Celite and 20 ml. of 0.4 N sodium hydroxide. Those residues showing a $\lambda_{\max}$. at 264 $m\mu$ and an apparent change in ϵ of not more than 300 units between 290 and 300 $m\mu$ were combined and recrystallized three times from ethanol. Pure 9-

dehydro-14-isoestrone separated as columnar crystals, $[\alpha]_D^{20} = +311^\circ$ (0.25 per cent in ethanol) and $+293^\circ$ (0.5 per cent in dioxane). The crystals acquired a purple coloration at 247° and melted to a blue liquid at $254-255^\circ$.

Analysis— $C_{18}H_{20}O_2$. Calculated. C 80.55, H 7.52
Found. " 80.63, " 7.47

500 γ of 9-dehydro-14-isoestrone benzenesulfonate, treated with dibromoquinonechloroimide reagent yielded no more color than the reagent blank. In the iron-Kober test the blue color developed was about equal to that given by equilin.

Identification of Equilenin—The crude equilenin-like compound obtained in the final portions of eluate from several chromatograms of isoequilin A was isolated by means of its picrate, and a portion of the purified ketosteroid was converted to its methyl ether by heating with 10 ml. of 2 N sodium hydroxide and 1 ml. of dimethyl sulfate for 1 hour. After extraction with chloroform from alkali, the dried ether was recrystallized twice from methanol, and melted at 194° . The melting point of pure *d*-equilenin methyl ether is 197° (14). The infra-red absorption spectrum of the methyl ether so obtained was identical with that of *d*-equilenin methyl ether (Curve A, Fig. 4), and exhibited none of the peaks characteristic of *dl*-isoequilenin methyl ether (Curve B, Fig. 4). (The methyl ether of *d*-isoequilenin, prepared by methylating pure *d*-isoequilenin, obtained by the method of Bachmann and Dreiding (14) from *d*-equilenin, yielded an infra-red spectrum identical with that given by the racemate.) The benzenesulfonate of another portion of the equilenin recovered from the chromatograms yielded an infra-red spectrum identical with that of *d*-equilenin benzenesulfonate. The spectrum of isoequilenin benzenesulfonate shows appreciable differences, and identification of the parent ketosteroid is also possible on the basis of this criterion.

Isomerization of 8- and 9-Dehydro-14-isoestrones—Pure 8-dehydro-14-isoestrone (20 mg.) was heated with 12 ml. of acetic acid and 8 ml. of hydrochloric acid under a reflux for 80 minutes. The cooled mixture was diluted with water and extracted with ether. The ether solution was washed with sodium carbonate and water, and evaporated to dryness. The residue, dissolved in alcohol and diluted, exhibited an ultraviolet $\lambda_{max.}$ at 264 m μ . Partition chromatography on a column of 31 gm. of Celite and 30 ml. of 0.4 N sodium hydroxide yielded about 5 mg. of 9-dehydro-14-isoestrone (identified by means of its chromatographic, ultraviolet, and infra-red properties) and about 2 mg. of isoequilenin (identified

by means of its ultraviolet absorption spectrum⁷ and by infra-red spectrophotometry of its benzenesulfonate), in addition to unchanged 8-dehydro-14-isoestrone.

Pure 9-dehydro-14-isoestrone (20 mg.), similarly treated, yielded small quantities (about 1 mg.) of purified 8-dehydro-14-isoestrone and isoequilenin.

Methyl Ether of 9-Dehydro-14-isoestrone—Pure 9-dehydro-14-isoestrone (30 mg.) was methylated by heating at 80° with 10 ml. of 2 N sodium hydroxide, and 1 ml. of dimethyl sulfate for 1 hour. The mixture was extracted with chloroform, and the solvent evaporated. The pink residue was recrystallized three times from ethanol, yielding 17 mg. of white tabular crystals, m.p. 143–144°.

Analysis— $C_{18}H_{22}O_2$. Calculated. C 80.81, H 7.85
Found. " 80.86, " 7.26

Methyl Ether of 8-Dehydro-14-isoestrone—The methyl ether of 8-dehydro-14-isoestrone was prepared by treating 30 mg. of the pure ketosteroid in the manner described for the 9 isomer. A blue color appeared in the alkali, and a red by-product in the chloroform extract. The methyl ether of 8-dehydro-14-isoestrone could not be freed of the tenacious, red by-product by repeated recrystallization from 50 per cent alcohol, or by chromatographic adsorption on alumina. After three recrystallizations the colored (red) bipyramidal crystals melted at 107–109°.

Acetate of 9-Dehydro-14-isoestrone—Pure 9-dehydro-14-isoestrone (20 mg.) was mixed with 2 ml. of dry pyridine and 0.5 ml. of acetic anhydride, and kept at 20° overnight. The solution was diluted with 50 ml. of water and extracted with two 15 ml. portions of chloroform. The combined chloroform extracts were washed and evaporated to dryness, and the residue recrystallized three times from diluted alcohol. Yield, 15 mg. of white needles, m.p. 124–125°.

Analysis— $C_{20}H_{22}O_2$. Calculated. C 77.38, H 7.15
Found. " 77.67, " 6.98

⁷ The ultraviolet absorption spectrum of isoequilenin differs from that of equilenin in that all of its maxima and minima are displaced 3 to 4 μ toward the shorter wave-lengths.

Equilenin, $\lambda_{\max}$	341	328	292.5	281	270
" $\lambda_{\min}$	332	303	288	275	264
Isoequilenin, $\lambda_{\max}$	338	325	289	277.5	267
" $\lambda_{\min}$	329	300	285	272	261

Acetate of 8-Dehydro-14-isoestrone—Pure 8-dehydro-14-isoestrone (20 mg.) was acetylated as described above. A pink color appeared in the solution, and the acetate was freed of the tenacious, more insoluble oily red by-product by repeated crystallization from 40 per cent alcohol. Yield, 9 mg. of white rod-shaped needles melting at 88–90° to a turbid liquid which became clear at 102°. Another sample, recrystallized from stronger alcohol, melted over a range of 102–105°. These discrepancies may be due to the presence of water of crystallization.

<i>Analysis</i> — $C_{20}H_{22}O_2$.	Calculated.	C 77.38, H 7.15
$C_{20}H_{22}O_2 \cdot \frac{1}{2}H_2O$.	"	" 75.21, " 7.26
	Found.	" 75.33, " 6.92

Sample dried to constant weight at 60°, 5 mm. of Hg.

β -9-Dehydro-14-isoestradiol—Pure 9-dehydro-14-isoestrone (30 mg.) was dissolved in 25 ml. of dry ether and shaken for 15 minutes at room temperature with 25 ml. of dry ether containing 1 gm. of lithium aluminum hydride. After decomposition of the excess lithium aluminum hydride with water and dilute hydrochloric acid, the diol was extracted with ether. The extract was washed successively with dilute acid and water, and evaporated to dryness. The residue was twice recrystallized from 50 per cent methanol. Yield, 22 mg. of white transparent bipyramidal crystals melting at 182–184°. When heated slowly, the crystal form apparently changed at about 100°, becoming opaque; when placed in a bath preheated above 150°, the crystals melted, solidified instantaneously, and remelted at 182–184°; $[\alpha]_D^{20} = +135^\circ$ (0.5 per cent in ethanol). Upon standing the crystals rapidly acquired a yellow coloration, and no satisfactory elementary analysis was obtained.

Isomerization of α -Didydroequilin (12)—Pure α -dihydroequilin⁴ (25 mg., m.p. 205°; $[\alpha]_D^{20} = +213^\circ$, 1 per cent in ethanol) was dissolved in 1 ml. of alcohol, mixed with 10 ml. of 0.5 N hydrochloric acid, and heated at 100° for 30 minutes. The cooled solution was extracted with ether and the solvent evaporated. The residue was dissolved in benzene and chromatographed on a column of 21 gm. of Celite and 20 ml. of 0.4 N sodium hydroxide. About 3 mg. of crystalline α -9-dehydro-14-isoestradiol were recovered in the anticipated benzene eluent fraction, and identified by means of its melting point (233–235°), its ultraviolet characteristics, and the infra-red absorption spectrum of its benzenesulfonate (Curve D, Fig. 2).

Isolation of 9-Dehydro-14-isoestrone from Commercial Mixed Estrogens—A commercial sample of "natural mixed estrogens," shown by infra-red analysis and by a dibromoquinonechloroimide reagent test after esterification to contain less than 1 per cent equilin, yielded a blue color in the iron-Kober test corresponding to 14 per cent equilin content. The mixture

(100 mg.) was dissolved in benzene and chromatographed on a column of 20 gm. of Celite and 20 ml. of 0.4 N sodium hydroxide. 9-Dehydro-14-isoestrone (6 mg.), identified by means of its melting point (254–256°) and ultraviolet and infra-red absorption characteristics, was recovered from the anticipated fractions of benzene eluate.

SUMMARY

Isoequilin A, prepared from equilin by acid isomerization, has been resolved into two major constituents: 8-dehydro-14-isoestrone, believed identical with Girard's hippulin, and a new estrogenic ketosteroid, 9-dehydro-14-isoestrone. The latter substance has been obtained also by the oxidation of Compound 3, first isolated by Hirschmann and Wintersteiner.

Compound 3 has been identified as α -9-dehydro-14-isoestradiol. Its β epimer has been prepared by the lithium aluminum hydride reduction of 9-dehydro-14-isoestrone.

By partition chromatography, 9-dehydro-14-isoestrone has been isolated from equine pregnancy urine extracts. It apparently occurs as an artifact produced by the isomerization of equilin during the acid hydrolysis of estrogen conjugates. Compound 3 is similarly obtained by the acid isomerization of α -dihydroequilin.

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TURNOVER AND OXIDATION OF BODY GLUCOSE IN NORMAL AND ALLOXAN-DIABETIC RATS*

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In a previous investigation we used C^{14} -labeled glucose to compare the rates of oxidation of carbohydrate in the normal and the alloxan-diabetic rat (1). Two methods were used. In one, glucose oxidation was based on the $C^{14}O_2$ evolved after a relatively large amount of the labeled glucose had been introduced intravenously into nephrectomized rats. The second method was designed to obtain a measure in the non-operated rat. In this case, glucose oxidation was determined from measurements of the $C^{14}O_2$ evolved and from the specific activity (s.a.) of plasma glucose. In obtaining the s.a. of plasma glucose, it was assumed that, at the early intervals after the injection of the labeled glucose, the total radioactivity of plasma was due to glucose.

In the present investigation we have improved these measurements of glucose utilization by determining the C^{14} content of blood glucose *per se*. We did this by preparing a phenylosazone of glucose from as little as 4 cc. of blood. Furthermore, we were careful to introduce only small amounts of glucose into the circulation. By these means we have now determined, for the normal and diabetic rat, (1) the size of the body pool of glucose, (2) the turnover time of body glucose, and (3) the turnover rate of body glucose. In addition, data are presented on the rate of oxidation of body glucose to CO_2 by the rat.

EXPERIMENTAL

Materials and Animals

Radioactive Glucose—The radioactive glucose was prepared photosynthetically according to Putman *et al.* (2). A single sample was used in this study, and we are indebted to Mr. S. Abraham for its preparation. The activity of the glucose was about 200,000 c.p.m. per mg. Its carbons were uniformly labeled with C^{14} .¹

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† Research Fellow of the United States Public Health Service, National Cancer Institute.

¹ Personal communication from Dr. W. Z. Hassid.

Animals and Their Treatment—The rats used were of two strains, Long-Evans and a highly inbred strain obtained from the genetic colony of this university. They were fed a diet consisting of 68.5 parts of wheat, 5 of casein, 10 of fish meal, 1.5 of salt, 5 of a fish oil, and 10 of alfalfa.

Alloxan Diabetes—The following procedure was used to induce diabetes. Each rat received a single intravenous injection (into the major saphenous vein) of 50 mg. of alloxan monohydrate² per kilo of body weight, as a 5 per cent aqueous solution. Animals that showed marked polyuria were placed in individual metabolism cages and daily measurements were made of their food and water intake, volume of urine, and the amount of glucose excreted. The concentration of glucose in whole blood was also determined in these rats in fed and fasted conditions. The blood was taken from the tail vein without anesthesia. Only rats that exhibited pronounced signs of diabetes were selected for study. They were fed the diet described above, supplemented, on alternate days, by an adequate vitamin mixture (3).

Methods

The methods employed for the determination of total expired CO₂ and for the analysis of blood and urine glucose have been described previously (1).

Preparation of Phenyllosazone of Whole Blood Glucose—Whole blood was deproteinated according to Somogyi's procedure (4) and the glucose content of the supernatant was determined. 10 or 20 cc. of the supernatant (equivalent to 1 or 2 cc. of whole blood) were used for the preparation of phenyllosazones, and to these was first added c.p. anhydrous glucose (as a rule, exactly 10.0 mg.) to serve as carrier. This was followed by the addition of 300 mg. of sodium acetate and 200 mg. of recrystallized phenylhydrazine hydrochloride (Eastman Kodak). The mixture was stirred well, heated on the steam bath for 4 hours, and allowed to cool gradually, first at room temperature and then in a refrigerator. The mixture was centrifuged and the supernatant was discarded. The precipitate was washed twice with 5 cc. of distilled water and two or more times with 15 cc. of warm ether to insure complete removal of aniline and excess hydrazine.

Purification of Phenylglucosazones—The phenylglucosazones were dissolved in 2 to 3 cc. of hot alcohol and the resulting solution was filtered, while hot, through Whatman No. 1 paper. The filtrate was diluted by the addition of an equal volume of hot distilled water. Crystallization of the phenylglucosazones was allowed to take place in this 50 per cent alcohol-water solution for 3 hours at room temperature and then overnight

² Obtained from Edean Company, East Norwalk, Connecticut.

at 4°. The phenylglucosazones were recrystallized three more times. They were redissolved each time in hot, 50 per cent alcohol, filtered while hot, and allowed to cool at room temperature.

The final product consisted of fine yellow needles. The supernatant was drawn off and the traces of alcohol and water were removed from the needles by drying them at 60° in a vacuum oven. The preparation of the phenylglucosazones was carried out in duplicate.

The decomposition points of the osazones were determined by means of an electrically heated block equipped with a magnifying lens (Fisher melting point apparatus). Samples that decomposed below 205°, or that appeared non-homogeneous, or that decomposed over a temperature range greater than that of a standard osazone prepared from pure D-glucose were recrystallized or discarded.

The phenylosazones were oxidized to CO₂, which was converted quantitatively to BaCO₃ and mounted on aluminum disks. The methods used for the oxidation, the mounting of the BaCO₃, and the determination of the C¹⁴ content of the plated BaCO₃ have been described elsewhere (1).

The phenylosazone of the glucose excreted by diabetic rats was prepared from an aliquot of clarified urine. Since plentiful amounts of glucose were present in each urine sample, no carrier was added. The procedure for isolation and purification of the urinary phenylglucosazone was identical with that used for blood.

Calculation of S.A. of Whole Blood Glucose—The s.a. of whole blood glucose refers to the per cent of the injected C¹⁴ recovered as blood glucose per mg. of blood glucose. The s.a. of whole blood glucose was calculated from the s.a. of the BaCO₃ obtained from the combustion of the glucosazones. The proper correction was made for the added carrier glucose.

Rate of Penetration of Radioactive Glucose into Red Blood Cell—In our earlier report (1), radioactive glucose was determined in *plasma*. Because only a small amount of plasma can be obtained from a rat, experiments were carried out to determine whether the s.a. of whole blood glucose is a reliable index of the s.a. of plasma glucose.

Blood was removed from normal and diabetic rats (as described below) 6, 10, 30, or 60 minutes after the injection of 8 to 10 mg. of labeled glucose. One-third of each blood sample was set aside, and the remainder was centrifuged for the separation of plasma. The proteins of whole blood were precipitated with acid ZnSO₄ and NaOH, and those of plasma with ZnSO₄ and Ba(OH)₂, as described by Somogyi (4, 5). The phenylosazone derivative of the glucose present in each fraction was prepared as described above, and the specific activities of these osazones were determined. The results are recorded in Table I.

6 minutes after the injection of the labeled glucose, the s.a. of plasma

glucose is considerably greater than that of whole blood glucose. This indicates that in 6 minutes, equilibrium between plasma glucose and red cell glucose is not reached. At the 30 minute interval, the values for s.a. of plasma and whole blood glucose were about equal in the diabetic rats. This also holds for normal rats that were sacrificed 1 hour after the injection of the C^{14} -glucose. Thus at 1 hour, the s.a. of whole blood glucose can be used as a reliable index of the s.a. of plasma glucose in the rat. For this reason, rats were not sacrificed earlier than 1 hour after the injection of the radioactive glucose.

TABLE I

Rate of Penetration of Intravenously Injected C^{14} -Glucose into Red Blood Cells of Normal and Diabetic Rats

Rat No *	Weight	C^{14} -Glucose injected	Interval after injection	Glucose concentration†		S.a.‡ of glucose	
				Plasma	Whole blood	Plasma	Whole blood
	gm.	c.p.m.	min.	mg. per cent	mg. per cent	$\times 10^3$	$\times 10^3$
Normal. N1	297	0.50×10^6	6	145	119	85.0	63.5
" N2	200	0.37×10^6	6	169	120	104	66.2
Diabetic. D1	168	0.48×10^6	10	428	331	51.5	48.4
" D2	170	0.48×10^6	10	558	407	39.0	33.2
" D3	278	1.27×10^6	30	788	502	10.6	10.4
" D4	266	1.00×10^6	30	454	328	18.8	19.1
Normal. N3	310	2.08×10^6	60	194	144	21.8	21.2
" N4	245	1.73×10^6	60	232	158	18.1	18.5

* All rats had access to food until sacrificed.

† The rats were anesthetized with ether and their chests opened, and blood was drawn from the ventricles.

‡ Specific activity, i.e., per cent of the injected C^{14} per mg. of glucose.

Results

Normal Rat

Female rats were employed because they are easy to catheterize. They had access to food up to the time of the experiment. During the experiment, they had access to water but not food. The rats were lightly anesthetized with freshly redistilled ether, and catheter tubing was inserted through the urethra into the bladder. Polythene plastic tubing (inside diameter 0.025 inch, wall thickness 0.004 inch) was used. The animals were placed in restraining cages (6) to prevent displacement of the catheter. The flow of urine from the catheter was quite uniform in each rat.

Immediately after insertion of the catheter and just before recovery from the effects of the ether, each rat received a single intravenous injection of labeled glucose. The amount injected varied from 12 to 18 mg.,

and each injection contained a total of 2 to 3×10^6 c.p.m. of C^{14} . The larger amounts were injected into rats that were sacrificed at the later intervals. The injection of 12 to 18 mg. of glucose provided sufficient activity for determination of the C^{14} contents of expired CO_2 and of the osazones prepared from blood and urine glucose. The blood sugar levels (the rats were not in the fasted state) were found normal 1 hour after the injection (130 to 180 mg. per cent).

The rat in its restraining cage was then placed in a glass chamber, well ventilated with CO_2 -free air, at $27-28^\circ$. Recovery from the anesthetic occurred within 10 minutes after the injection of the glucose.

Expired CO_2 was trapped in NaOH. Samples of urine and CO_2 were collected at half hour intervals.

Although no glucose was found in the urine of the *normal* rats, they were catheterized to insure experimental conditions identical with those for the *diabetic* rats.

Measurements Derived from S.A.-Time Curve of Blood Glucose

At 1, 2, 2.5, or 3 hours after the injection of the labeled glucose, the rats were lightly anesthetized with ether. Their chests were opened, and 4 to 8 cc. of blood were withdrawn from the left ventricle directly into a heparinized syringe. The specific activities of blood glucose were determined as described above. Individual values for specific activities of blood glucose have been plotted on semilog paper with a linear time axis (Fig. 1). From theoretical considerations discussed elsewhere (7), an exponential fall in s.a. of blood glucose is to be expected in an experiment of the type described in this study.³ Therefore, we have assumed that the data, when plotted as shown, can be represented by a straight line, and we have used the method of least squares to draw the best line.

It is apparent from this curve that the expected initial rapid fall in s.a. of blood glucose (not shown since blood was not taken before 1 hour), the result of mixing of circulating labeled glucose with non-labeled extravascular glucose, must have been complete before 1 hour had elapsed.⁴ If mixing had not been complete by that time, the curve shown in Fig. 1 would not approximate a straight line so closely.

³ In similar experiments carried out in dogs, we are able to obtain several samples of plasma glucose from the *same* animal. The fall in s.a. of plasma glucose with time also appeared to be exponential in nature. The deviation of the points from the straight line in the case of the dog, as measured by the *standard error of estimate* (8), was about 50 per cent less than that of the composite rat curve shown in Fig. 1. A greater deviation is perhaps to be expected when observations are made on a large number of animals rather than on a single animal.

⁴ It is of interest to compare the glucose curve shown in Fig. 1 with that obtained by Flexner *et al.* for heavy water (9). Mixing between vascular and extravascular water, as judged by the rapid fall in DHO concentration in water of blood with time, was complete in 10 minutes.

Determination of Size of Glucose Body Pool in Dynamic Equilibrium with Circulating Glucose—The exponential decrease in s.a. of blood glucose between 1 and 3 hours (Fig. 1) is the result of the removal of a constant fraction of the total glucose body pool per unit of time. It represents the chemical turnover of the glucose pool when the size of this pool re-

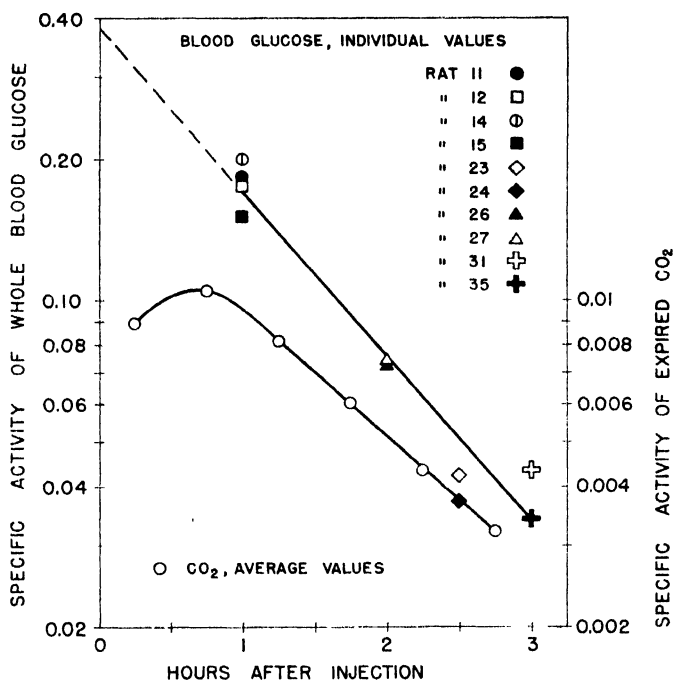


FIG. 1. S.a.-time curves of blood glucose and expired CO₂ for normal rats. Each glucose value is the average of duplicate determinations made on the blood of a single normal rat at the time it was sacrificed. S.a. of whole blood glucose refers to per cent of the injected C¹⁴ recovered as blood glucose per mg. of glucose. The data for the CO₂ curve were obtained from a group of ten normal rats and each point is the average of four to ten separate determinations. The s.a. of expired CO₂ refers to per cent of injected C¹⁴ recovered as BaCO₃ per mg. of BaCO₃.

mains constant; *i.e.*, when the animal is in the steady state with respect to its glucose metabolism. The size of the glucose pool that had become labeled can be determined by extrapolation of the s.a.-time curve of blood glucose to zero time. In the normal rat, this pool amounts to 260 mg. This glucose pool represents more than circulating glucose; indeed, it represents body glucose in equilibrium with circulating blood glucose, *i.e.*, extravascular as well as vascular. The measurements made here involve only blood glucose, but since that is in rapid equilibrium with the larger glucose pool, it is the fate of the glucose in this larger pool that

is, in effect, being measured. For purposes of discussion we shall refer to this pool as *body glucose*.

The anatomical interpretation of the glucose compartment here designated *body glucose* is not without difficulty. Glucose can be stored in a form other than free glucose, *i.e.*, as glycogen. To what extent liver glycogen participates in the glucose pool under consideration cannot be stated. The fact that the amount of glucose in this pool represents more than that present solely in extracellular spaces is borne out by several observations. If it is assumed that the concentration of glucose in the extravascular spaces is the same as that in the blood stream, the *glucose space* would amount to about 130 cc., or about 65 per cent of the animal's body weight. The volume of the glucose space appears to be about the same as that occupied by body water (10). Secondly, expired $C^{14}O_2$ is formed within minutes after the intravenous injection of C^{14} -glucose.

This observation indicates that the injected C^{14} -glucose has, within a matter of minutes, entered the cell. And, finally, the data recorded in Table I indicate that equilibrium between plasma glucose and red cell glucose is nearly complete shortly after the introduction of labeled glucose into the circulation.

Turnover Time of Body Glucose (t_t)—This refers to the time required for the disappearance of an amount of glucose equal to body glucose. $t_t = 1.44t_1$ where t_1 is the time required for the s.a. of blood glucose to decrease by one-half. t_1 , as shown in Fig. 1, is 0.85 hour. Therefore, $t_t = 1.23$ hours.

Turnover Rate of Body Glucose—Turnover rate refers to the amount of body glucose turned over per unit of time. This is obtained by dividing the total amount of glucose in the glucose pool by the turnover time. The turnover rate of body glucose is 212 mg. per hour. Since the average weight of the ten rats used in this experiment was 205 gm., the turnover rate of body glucose amounts to about 100 mg. per hour per 100 gm. of body weight.

S.A.-Time Curve of Expired CO_2

The s.a. of expired CO_2 is here defined as the percentage of the injected C^{14} recovered as $BaCO_3$ per mg. of $BaCO_3$. Average values have been plotted on a semilog scale, in Fig. 1, at the mid-point of the interval under consideration. The data were obtained from a group of ten normal rats. Each CO_2 point in Fig. 1 represents the average of four to ten separate determinations made on as many rats. The rats were sacrificed 1, 2, 2.5, or 3 hours after the injection of the labeled glucose; thus two to six determinations were made on each rat. The average weight of this group of rats⁵ (Rats N11 to N13, N21, N22, N24, N31 to N33, and N36) was 181 gm.

⁵ In the case of the glucose curve shown in Fig. 1, each value is the average of duplicate determinations made on the blood of a single rat at the time it was sacrificed.

The s.a. of expired CO_2 rose to a maximum at an early period and then fell in a simple exponential manner. The peak of the curve appeared between 30 and 60 minutes after the injection. The time at which this peak is observed corresponds with the time that maximum labeling of the CO_2 body pool occurs. At this time, the s.a. of the CO_2 body pool has attained a value equal to that of the CO_2 precursors (7).

TABLE II

Expired CO_2 Recovered As Mg. of BaCO_3 from Normal Rats

Each rat was injected intravenously with 12 to 18 mg. of C^{14} -glucose

Rat No.	Weight	Expired CO_2 recovered as BaCO_3 for each interval					
		0-0.5 hr.	0.5-1.0 hr.	1.0-1.5 hrs.	1.5-2.0 hrs.	2.0-2.5 hrs.	2.5-3.0 hrs
	gm.	mg.	mg.	mg.	mg.	mg.	mg.
24	207	786	1355	1110	1185	1015	
33	180	586	1115	1360	1240	1245	1340
34	203	1010	1200	1070	1000	950	1000
36	165	1000	1000	980	970	810	790
38	210	602	979	810	764	847	967
41	180	625	840	860	700	806	688
42	180	392	646	710	912	923	690
Average....	189	714	1020	990	970	940	913
Average BaCO_3 per 100 gm. body weight, mg.. .. .		378	540	525	515	497	483

Total CO_2 Expired

Data were obtained from the seven normal rats recorded in Table II. The average body weight of this group was 189 gm. Each rat was kept in a glass cage, without disturbance, for 3 hours. Expired CO_2 was collected for each half hour interval and converted to BaCO_3 . The yields of BaCO_3 for each interval are recorded in Table II. With the exception of the first half hour, the amounts of CO_2 collected per half hour were fairly constant.

Some Assumptions Involved in Use of S.A.-Time Curves of CO_2 and Blood Glucose For Calculation of Amount of Body Glucose Oxidized to CO_2

The amount of body glucose oxidized to CO_2 per hour can be estimated from the following data: (1) the s.a.-time curve of blood glucose; (2) the

s.a.-time curve of expired CO_2 ; and (3) the total amount of CO_2 expired per hour. The amount of glucose oxidized to CO_2 per hour is given by

$$\frac{\text{(Average s.a. BaCO}_3 \text{ derived from expired CO}_2 \text{ during hr.)}}{\text{(mg. BaCO}_3 \text{ derived from expired CO}_2 \text{ for that hr.)}} \quad (1)$$

Average s.a. of blood glucose for that hr.

To permit the calculation to be made, the following conditions must hold: (1) the injected labeled glucose must be completely mixed with body glucose; (2) the s.a. of expired CO_2 must represent the s.a. of the body pool of CO_2 ; (3) in the series of oxidation processes beginning with the removal of labeled glucose from the circulation and ending with the exhalation of CO_2 , the one process measured here (disappearance of the glucose) should be the rate-determining step; and (4) the rate of conversion of glucose to CO_2 should be constant.

Mixing of Injected Glucose with Body Glucose—For the estimation of glucose oxidation shown below, we used s.a. values of blood glucose obtained no earlier than 1 hour following injection. For reasons presented above, it is reasonable to conclude that "complete" mixing between the injected labeled glucose and body glucose had occurred within 1 hour. Moreover, the exponential nature of the curve (Fig. 1) shows that, during the intervals between 1 and 3 hours, the amount of radioactivity returning to the circulation as newly synthesized glucose is negligible.

Body Pool of CO_2 —We have assumed that expired CO_2 is derived directly from the body pool of CO_2 (most of which is bicarbonate), so that the s.a. of the CO_2 collected is identical with the s.a. of the body pool of CO_2 .

Rate-Determining Step in Conversion of Plasma Glucose to Expired CO_2 —In order to calculate the glucose oxidized to CO_2 at any given instant, from the two curves shown in Fig. 1, it is necessary to assume that the first in the series of reactions concerned in chemical turnover of body glucose, namely, the removal of labeled glucose from the circulation, is the rate-determining step. We have assumed that, once the glucose molecule is removed from the body pool, it is attacked immediately and converted to CO_2 so rapidly that the s.a. of expired CO_2 at any instant can, for practical purposes, be related directly to the s.a. of the body glucose at the same instant. Since C^{14}O_2 is expired within a few minutes after injection of C^{14} -glucose, this appears to be a reasonable assumption.

In a first-order reaction, the rate of disappearance of the reactant is proportional to the slope of the curve representing the logarithm of the concentration of the reactant as a function of time. The s.a.-time curve for circulating glucose tends to parallel the s.a.-time curve for expired

CO₂ (for the interval 1 to 3 hours, Fig. 1). The fact that the slopes of the two curves are approximately the same indicates that the rate of appearance of C¹⁴O₂ is dependent upon the rate of disappearance of circulating labeled glucose. This finding would appear to justify our assumption that the rate of disappearance of glucose from the circulation is the rate-determining step in the conversion of body glucose to CO₂.

Constancy of Rate of Conversion of Body Glucose to CO₂—For reasons outlined in the preceding sections, the calculation for the rate of conversion of body glucose to CO₂ is based on data obtained for the interval 1 to 3 hours after the injection of C¹⁴-glucose. The amounts of glucose oxidized to CO₂ per unit of time between 1 and 3 hours, as judged by our calculation, are shown below to be approximately constant.

Values for Glucose Oxidation in Normal Rat

Calculation of Amount of Body Glucose Oxidized to CO₂—Values for the amounts and specific activities of expired CO₂ are recorded in Table II and Fig. 1. The s.a. of expired CO₂ and of blood glucose was read from Fig. 1 at the mid-point of the interval under consideration; the s.a. value obtained at this point represents the average s.a. of the interval.

The numerator in expression (1) represents the per cent of the injected C¹⁴ recovered in the expired CO₂ for each hour. Thus, the calculation presented here is identical with that used in an earlier study (1), except that, for reasons presented above, it is based on data obtained for intervals between 1 and 3 hours.

By substitution in Expression 1 the amount of glucose oxidized for the 1 hour interval between the 1st and 2nd hour after injection was found to be 120 mg.

The amount of glucose oxidized between the 2nd and 3rd hours was 134 mg. The average for the entire interval (1 to 3 hours) is 127 mg. per hour. The average weight of the twenty-one normal rats (Fig. 1, Table II, and text) used in this study was 188 gm. These rats, therefore, oxidized 69 mg. of glucose per hour per 100 gm. of body weight.

Per cent of Expired CO₂ Carbon Derived from Body Glucose Carbon—The per cent of the expired CO₂ carbon derived from body glucose carbon at any instant is given by

$$\frac{\text{S.a. of CO}_2 \text{ carbon at that instant}}{\text{S.a. of body glucose carbon at same instant}} \times 100$$

The values for this expression at 1, 2, and 3 hours are given in Table III. The average of the three values is 44.7 per cent.

TABLE III

Per Cent of Expired CO₂ Carbon Derived from Oxidation of Glucose Carbon in Normal Rats

Each rat was injected intravenously with 12 to 18 mg. of C¹⁴-glucose.

Time after injection hrs.	S.a. of blood glucose		S.a. of expired CO ₂		Expired CO ₂ carbon derived from oxidation of glucose carbon <i>per cent</i>
	Per mg. glucose $\times 10^3$	Per mg. glucose carbon $\times 10^3$	Per mg. BaCO ₃ $\times 10^3$	Per mg. BaCO ₃ carbon $\times 10^3$	
1	17	42.5	9.7	160	37.6
2	7.6	19	5.1	84.2	44.3
3	3.4	8.5	2.7	44.5	52.3

TABLE IV

History of Alloxan-Diabetic Rats Used in This Study

Rat no.*	Strain	Rat weight		Duration of diabetes	Degree of diabetes			
		When injected	When sacrificed		Range, urine flow in 24 hrs.†	Maximum urine sugar for 24 hr. sample†	Fasting blood sugar‡	Blood sugar at the time of sacrifice (non-fasted)
		gm.	gm.	days	cc.	gm.	mg. per cent	mg. per cent
D11	G. C.	220	177	34	45- 80	7.0	300	615
D12	"	186	180	40	40- 70	4.5	236	575
D13	"	266	237	48	40- 80	8.0	200	600
D14	L. E.	178	164	30	35- 60	5.3	246	616
D21	G. C.	244	162	33	60-115	5.9	290	500
D22	L. E.	220	195	23	40- 80	6.5	235	800
D31	"	166	144	41	35- 65	4.5	435	525
D32	G. C.	90	118	38	50- 75	6.2	222	676
D36	L. E.	132	180	63	45- 70	5.9	450	
D37	"	134	200	64	60-110	7.4	215	
D38	G. C.	224	242	43	75-100	5.2		401
D39	L. E.	120	160	52	60- 95	7.8		374

* All the rats used were females. G. C. refers to the genetic colony strain, L. E. to the Long-Evans strain.

† Determined every day for duration of diabetes.

‡ Determined 4 to 6 days before experiment. Blood was obtained from the tail vein without anesthetizing the rat.

Diabetic Rat

The history of the twelve diabetic rats used is recorded in Table IV. Diabetes had been present in the rats for periods of at least 3 weeks to as long as 2 months when the experiment was started. Two values for blood sugar are given for each rat: (1) that found at the end of the experi-

mental period, the rat not in the fasted state at this time; and (2) a 24 hour fasted value determined, as a rule, 4 to 6 days before the beginning of the experiment. The values for (1) ranged from 374 to 800 mg. per cent; those for (2) from 200 to 450 mg. per cent. Daily glucose excretion ranged from 4 to 8 gm. per rat.

The experimental procedures and treatment of the diabetic rats were identical with those described for normal rats. Following the intravenous injection of the labeled glucose, the rat in its restraining cage was placed in the chamber, and expired CO_2 and urine were collected for each half hour interval. The rats were sacrificed at various times (1, 2, or 3 hours) thereafter and blood was removed for the preparation of phenylglucosazones.

S.A.-Time Curve of Blood Glucose—The values for the s.a. of blood glucose were obtained from phenylglucosazones in the manner described above. The data shown in Fig. 2 were obtained from seven diabetic rats whose average weight was 163 gm. Each glucose value shown in Fig. 2 represents the average of duplicate determinations on a single blood sample obtained from a rat at the time it was sacrificed.

S.A.-Time Curve of Urinary Glucose—The total amount of glucose excreted for each half hour interval was determined by its reducing value. The amount of glucose excreted per rat was quite variable, ranging from 80 to 135 mg. per hour per 100 gm. of body weight (Table V).

From one to six urine samples were obtained from each rat during the course of the experiment. Phenylglucosazones were prepared from each urine sample and purified as described above for blood glucose. The specific activities of urinary glucose were calculated, and the value for each sample was plotted on semilog paper (Fig. 2) at the mid-point of the interval under consideration.

The s.a. values for both blood and urine glucose appear to fall on the same straight line. The best fit for all values was obtained by the method of least squares. This coincidence between the s.a. of urine glucose and that of blood glucose was reported earlier (1).

Calculation of Body Pool of Glucose in Dynamic Equilibrium with Blood Glucose in Diabetic Rat—The zero time value for s.a. in the glucose curve shown in Fig. 2 is 23.4×10^{-2} . Total body glucose is, therefore, 427 mg.

Turnover Time of Body Glucose—The half time of the glucose curve shown in Fig. 2 is 0.85 hour; the turnover time is, therefore, 1.23 hours.

Turnover Rate for Body Glucose—The turnover rate for the diabetic rat is 347 mg. per hour. This amounts to 213 mg. of glucose per hour per 100 gm. of body weight. In the diabetic rat, in contrast to the normal, a large part of the glucose turned over is excreted in the urine. The amounts excreted are recorded in Table V.

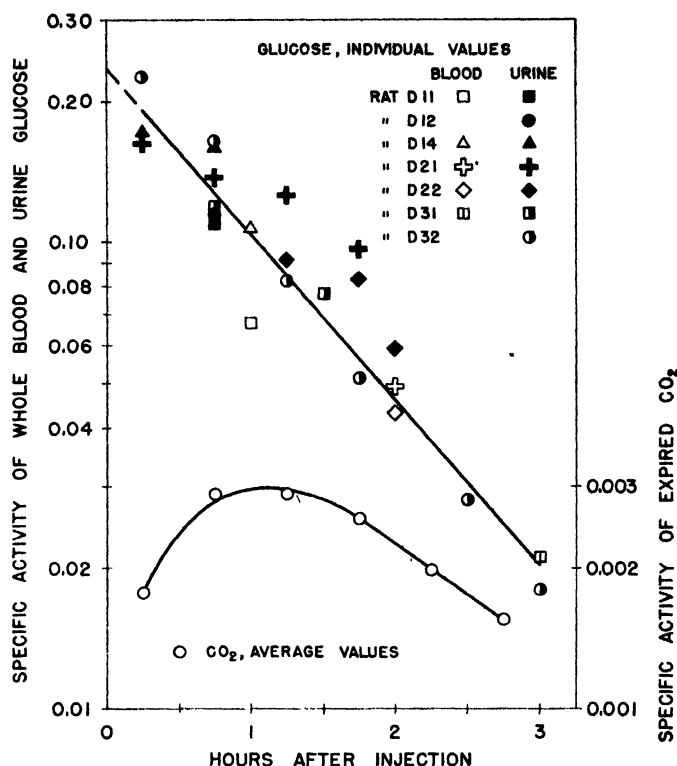


FIG. 2. S.a.-time curves of blood and urine glucose and of expired CO₂ for alloxan-diabetic rats. Each blood glucose value is the average of duplicate determinations made on the blood of a single rat at the time of sacrifice. Each diabetic rat contributed from one to six urine-s.a. values, depending on the time of its sacrifice after injection of the labeled glucose. The data for the CO₂ curve were obtained from a group of five alloxan-diabetic rats, and each point is the average of five separate determinations on the same number of rats.

TABLE V

Glucose Excreted in Urine of Diabetic Rats after Injection of 12 to 18 Mg. of Radioactive Glucose

The duration of the experiment was 3 hours. Urine was collected at half hour intervals. Each glucose value recorded below is the average of the six separate determinations.*

Rat No.	D11	D12	D21	D22	D31	D32
Mg. per hr. per 100 gm. body weight.....	80	120	90	115	115	135

Total CO₂ Expired and S.A.-Time Curve of Expired CO₂—Radioactive glucose was injected into five diabetic rats (Rats D14 and D36 to D39).

The total CO_2 expiration was measured in four of these rats, and the six half hour values for each rat are recorded in Table VI. The C^{14}O_2 data were obtained from all five rats. As noted above, the rats were not disturbed during the collection of CO_2 .

The s.a. of expired CO_2 was determined, as described above, and the value was plotted on a semilog scale in Fig. 2, at the mid-point under

TABLE VI

Expired CO_2 Recovered As Mg. of BaCO_3 from Diabetic Rats

Each rat was injected intravenously with 12 to 18 mg. of C^{14} -glucose.

Rat No.	Weight	Expired CO_2 recovered as BaCO_3 for each interval					
		0-0.5 hr.	0.5-1.0 hr.	1.0-1.5 hrs.	1.5-2.0 hrs.	2.0-2.5 hrs.	2.5-3.0 hrs.
	gm.	mg.	mg.	mg.	mg.	mg.	mg.
D36	180	510	795	825	1040	890	1020
D37	200	622	990	1100	1510	1050	920
D38	242	814	1470	1660	1410	1420	1280
D39	160	900	1090	930	825	950	890
Average....	195	710	1080	1120	1190	1070	1020
Average BaCO_3 per 100 gm. body weight, mg.		364	553	573	610	548	522

TABLE VII

Per Cent of Expired CO_2 Carbon Derived from Oxidation of Glucose Carbon in Diabetic Rats

Each rat was injected intravenously with 12 to 18 mg. of C^{14} -glucose.

Time after injection	S.a. of blood glucose		S.a. of expired CO_2		Expired CO_2 carbon derived from oxidation of glucose carbon
	Per mg. glucose	Per mg. glucose carbon	Per mg. BaCO_3	Per mg. BaCO_3 carbon	
hrs.	$\times 10^3$	$\times 10^3$	$\times 10^3$	$\times 10^3$	per cent
2	4.6	11.5	2.3	38	33
3	2.0	5.0	1.4	23.1	46.2

consideration. Each value recorded in Fig. 2 is the average of five s.a. determinations, one from each of the five rats. The average weight of this group of rats was 193 gm.

The s.a. was maximum somewhere between 45 and 75 minutes after the injection and thereafter decreased exponentially.

Body Glucose Oxidized to CO_2 —Calculations were made for the following intervals: 1.5 to 2.0 hours, 2.0 to 2.5 hours, and 2.5 to 3.0 hours. The values found were 98, 102, and 108 mg. per hour, respectively. The average value was 103 mg. per hour. Since the weights of the diabetic rats

differed from the normal, we have expressed the oxidation of glucose on a body weight basis. The average weight of the entire group of twelve diabetic rats (Table IV) was 175 gm. Thus the rate of glucose oxidation was 59 mg. per hour per 100 gm. of body weight.

Per cent of Expired CO₂ Carbon Derived from Oxidation of Glucose Carbon—The per cent of CO₂ carbon derived from glucose carbon has been calculated as described above and found to be 40 per cent (Table VII). This value agrees closely with that found for the normal rat, namely, 45 per cent.

DISCUSSION

In the present investigation an attempt has been made to provide some measures of glucose utilization in the intact animal. Those discussed are turnover time of body glucose, turnover rate of body glucose, and the rate of conversion of body glucose to CO₂. These three measures of glucose utilization were obtained by introducing uniformly labeled C¹⁴-glucose directly into the circulation. The subsequent fall in the s.a. of blood glucose with time provided the data from which the turnover measurements were calculated, whereas the rates of body glucose oxidation were obtained from a study of the s.a.-time curves of blood glucose and expired CO₂.

Normal Rat—Pertinent to the interpretation of the values obtained by these procedures is the amount of labeled glucose introduced directly into the circulation. Ideally, the amount should not appreciably increase the total glucose content of the animal. Extrapolation of the s.a.-time curve of blood glucose to zero time, in the experiments carried out with 12 to 18 mg. of labeled glucose, reveals that the labeled glucose becomes part of a compartment, or pool, containing 260 mg. of glucose. This is about 15 times the amount of glucose contained in the rat's circulation. It therefore becomes clear that glucose undergoing utilization at any time is in rapid equilibrium with the glucose of a dynamic pool that includes not only blood glucose but also glucose in extracellular spaces and in intracellular spaces of many tissues.

It is apparent from the above considerations that the introduction of only 12 to 18 mg. of the labeled glucose into the circulation of the *normal* rat did not appreciably increase the glucose content of the dynamic pool of glucose. The values for glucose utilization obtained by using this dose may therefore be regarded as representing what is occurring in the steady state with respect to carbohydrate metabolism. In the experiments under consideration, this refers to the unfasted state because the rats had access to food up to the time they were injected with the labeled glucose.

The data summarized in Table VIII present, for the *intact* normal rat,

an analysis of glucose utilization. The rat turns over an amount of glucose equal to that contained in its body pool about every 70 minutes, and this represents a turnover of approximately 100 mg. of glucose per hour per 100 gm. of body weight. According to this figure, a normal rat weighing 200 gm. turns over, per day, approximately 5 gm. of glucose. About 67 per cent of the glucose turned over is oxidized to CO_2 (Table VIII). The 34 mg. designated "unaccounted for" in Table VIII are, of course, converted to fatty acids, glycogen, etc. It is also shown here that, in the rat ingesting a diet containing about 60 per cent carbohydrate, 45 per cent of the CO_2 carbon in the expired air is derived from the oxidation of glucose.

It is of interest to compare this breakdown of glucose utilization in the normal rat with that presented recently by Stetten (11). According to

TABLE VIII

Breakdown of Glucose Utilization in Normal and Diabetic Rats

Each rat was injected intravenously with 12 to 18 mg. of labeled glucose, an amount which, as explained in text, does not disturb the steady state of the rat.

Type	Body pool of glucose	Turnover time of body glucose	Turnover rate of body glucose	Glucose excreted	Oxidation to CO_2 of body glucose	Glucose unaccounted for
	mg. per 100 gm. body wt.	min.	mg. per hr. per 100 gm. body wt.	mg. per hr. per 100 gm. body wt.	mg per hr. per 100 gm. body wt.	mg per hr. per 100 gm body wt
Normal..	127	74	103	0*	69	34
Diabetic.	262	74	213	80-135	59	19-78

* * A catheter was inserted into bladder of each normal rat and kept there during the 3 hours of the experiment. No glucose was found in urine so collected.

this worker, about one-third of total body glucose is used for fatty acid formation, about one-thirtieth for glycogen formation, while the bulk of the remainder is ultimately degraded to CO_2 .

Diabetic Rat—Our findings on glucose utilization by the intact, alloxan-diabetic rat are also summarized in Table VIII. The body glucose pool in the diabetic rat was, of course, larger than normal, but, interestingly enough, the time required for complete renewal of this larger pool of glucose was also about 70 minutes. Thus, glucose is being turned over in the diabetic rat at a rate about twice that in the normal rat. In the case of a 200 gm. diabetic rat this would amount to about 10 gm. in 24 hours. It should not be inferred, however, that *all* of this *extra* glucose arises by way of gluconeogenesis, for the diabetic rat ingests about twice as much food as the normal rat.

The rate of oxidation of glucose by the alloxan-diabetic rat was about

60 mg. per hour per 100 gm. of body weight. This rate of glucose *oxidation* is somewhat less than that found for the normal rat. The difference from normal, however, is not of sufficient magnitude to permit us to conclude that impaired glucose *oxidation* is the major metabolic defect in the intact alloxan-diabetic rat. In this respect the observations recorded here support our earlier conclusions (1).

It should not be inferred from the findings presented here that glucose *utilization* is not impaired in the alloxan-diabetic rat. It was shown by Stetten and his associates, with the aid of D_2O , that glucose utilization for both lipogenesis and glycogenesis is greatly depressed in the rat made diabetic by alloxan injections (12, 13). Some experiments carried out recently in this laboratory also bear on this point (14). With the aid of C^{14} -labeled glucose, we have studied the conversion of glucose to fatty acids and CO_2 by surviving liver slices. The results obtained have clearly demonstrated that the liver of the alloxan-diabetic rat loses almost completely its capacity to use carbohydrate for fatty acid synthesis. The conversion of the added C^{14} -glucose to CO_2 , by the diabetic liver, was also depressed, but in all cases the impairment in glucose utilization for lipogenesis was more pronounced than that involving the oxidation of glucose to CO_2 .

SUMMARY

1. The size of the body glucose pool, turnover time, and turnover rate of this body pool, and the rate of oxidation of body glucose to CO_2 have been estimated in the intact rat. These estimates of glucose utilization were obtained by introducing small amounts of C^{14} -labeled glucose directly into the circulation. The isolation of the phenylosazone of the glucose present in small amounts of blood made possible the direct determinations of the specific activities of blood glucose.

2. The normal rat has a body pool of glucose (*i.e.*, glucose in rapid equilibrium with the glucose undergoing utilization) amounting to about 130 mg. per 100 gm. of body weight. An amount of glucose equal to that contained in the body pool is completely turned over about every 70 minutes. Glucose is therefore being turned over in the normal rat at a rate of about 100 mg. per hour per 100 gm. of body weight. About 67 per cent of the glucose turned over is oxidized to CO_2 . Approximately 45 per cent of the CO_2 carbon in expired air is derived from glucose carbon.

3. The body pool of free glucose is greatly increased in the alloxan-diabetic rat and amounts to about 260 mg. per 100 gm. of body weight. An amount equal to this is completely renewed every 70 minutes, and, consequently, the diabetic rat turns over body glucose at a rate of 210

mg. per hour per 100 gm. of body weight. About 60 mg. of the glucose turned over is converted to CO_2 . About 40 per cent of the CO_2 carbon in expired air is derived from glucose carbon.

4. The rate of conversion of body glucose to CO_2 is slightly reduced in the diabetic rat. Impaired oxidation of glucose to CO_2 does not appear to be the major metabolic defect in the *intact* alloxan-diabetic rat.

5. The question of defective utilization of glucose, particularly in the liver, in the alloxan-diabetic rat is discussed.

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BIOSYNTHESIS OF LABELED FATTY ACIDS AND CHOLESTEROL IN EXPERIMENTAL DIABETES*

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Within the past few years, substantial evidence has been advanced by Stetten and Boxer (1) and Stetten and Klein (2) that there is marked impairment of fat synthesis in the diabetic animal. These investigators have shown by studies with deuterium that from 35 to 50 per cent of ingested carbohydrate is rapidly converted to fat in the normal animal, whereas, in the diabetic animal, this conversion of carbohydrate to fat is markedly inhibited. Gurin, Delluva, and Wilson (3) have reported that the phlorhizinized rat converts C^{14} -labeled lactate to fat and ketone bodies to the extent of nearly 50 per cent of the administered lactate. The administration of insulin to normal rabbits markedly increases fat synthesis in the liver (4). Recently we reported (5) that insulin stimulates long chain fatty acid synthesis from acetate *in vitro* by surviving rat liver slices. The investigations reported here were undertaken to study the behavior of liver tissue from diabetic animals.

EXPERIMENTAL

Complete details of the experimental procedure have been previously reported (6). Essentially, slices of liver tissue were incubated in a Warburg apparatus in Krebs-Ringer bicarbonate buffer solution in the presence of a short chain fatty acid substrate labeled with C^{14} . In order to study the effect of insulin, 1 mg. of electrophoretically homogeneous insulin¹ was dissolved in 1 ml. of water, the pH adjusted to 7.4, and the solution added to the incubating medium. Following incubation, the liver slices were digested with alcoholic KOH and fatty acids isolated according to the usual procedures. In order to remove any unutilized radioactive substrate, the long chain fatty acids were precipitated with a copper-lime mixture (7) which effectively precipitates all those fatty acids whose chain

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¹ We wish to express our thanks to Sharp and Dohme, Inc., for the insulin.

lengths are longer than 10 carbon atoms, whereas those of shorter length remain in the supernatant. After appropriate washing and reprecipitation (6), the long chain fatty acids were isolated, dried, weighed, and analyzed for radioactivity. The radioactivity measurements were made as described previously (6). The non-saponifiable fraction (in ether) was washed repeatedly with an aqueous solution of non-labeled sodium acetate, and the cholesterol precipitated from alcohol as the digitonide which was washed according to the modification of Srere, Chaikoff, and Dauben (8). That this procedure effectively frees the isolated cholesterol from contaminating substrate is attested by our failure to find any demonstrable radioactivity in cholesterol recovered from homogenized liver tissue incubated with carboxyl- or methyl-labeled acetate in phosphate or bicarbonate buffer. The cholesterol digitonide was analyzed for cholesterol by adapting the procedure of Schoenheimer and Sperry (9) for use with the Beckman quartz spectrophotometer at 620 $m\mu$.

Rats were made diabetic by intraperitoneal injection of a 2 per cent aqueous solution of alloxan (160 mg. per kilo). The blood sugar was analyzed from a sample taken from the tail vein according to the method of Polis and Sortwell (10). The pancreatectomized cats were very generously supplied by Dr. A. Milman and Dr. F. D. W. Lukens. Carboxyl-labeled octanoate was prepared as previously described (6).

Results

Whereas liver slices from normal rats of the Wistar strain rapidly convert labeled acetate or labeled short chain fatty acids to long chain fatty acids (or fats), this was found not to be the case with alloxanized rats. Liver slices from rats rendered diabetic with alloxan showed a marked reduction in the amount of acetate or octanoate incorporated into long chain fatty acids. This failure of fatty acid synthesis was not reversed by the addition of insulin to the incubating medium (Table I), whereas insulin caused a definite stimulation of fatty acid synthesis in liver slices of normal rats (6). Furthermore, the addition of glucose plus insulin, fructose-6-phosphate and insulin (with or without added α -ketoglutarate), oxalacetate, or α -ketoglutarate alone or with insulin failed to restore the ability of the diabetic liver slice to synthesize fatty acids (Table I). In addition, no stimulating effect of insulin could be obtained with diabetic liver slices, either upon addition of a boiled supernatant derived from homogenized normal liver or of a whole homogenate of normal liver.

Because of the difficulties encountered in performing total pancreatectomy in the rat, we used liver slices from fed and fasted (48 hours) normal cats, as well as from fasted pancreatectomized cats. In the case of the normal or fasted cat, the addition of amorphous insulin to the incubating

medium caused an increase in the amount of acetate incorporated into fatty acids (Table II). On the other hand, liver slices from pancreatec-

TABLE I

Conversion of Acetate and Octanoate to Long Chain Fatty Acids by Liver Slices from Normal and Diabetic Rats

Ex- peri- ment No.*	Blood glucose	Substrate		In- sulin	Recovered long chain fatty acids			
		Formula	Radio- activity admin- istered		Per 2.5 gm. liver slices	Radio- activ- ity	Per cent admin- istered radio- activ- ity	Sub- strate incor- porat- ed†
	<i>mg. per 100 ml.</i>		<i>c.p.m.</i>		<i>mg.</i>	<i>c.p.m.</i>		<i>μM</i>
1	Normal	C ¹⁴ H ₃ COONa	17,000	—	55	5800	34	25
	"	"	17,000	+	50	8500	50	37
2	"	CH ₃ (CH ₂) ₆ C ¹⁴ OONa	5,800	—	40	520	9	3.8
	"	"	5,800	+	40	720	12.5	5.2
3	"	C ¹⁴ H ₈ COONa	56,000	+	75	8600	15.3	37
	"	CH ₃ (CH ₂) ₆ C ¹⁴ OONa	19,000	+	77	1200	6.3	8.6
4†	450	C ¹⁴ H ₃ COONa	17,000	—	150	350	2.1	1.5
	450	"	17,000	+	135	310	1.8	1.4
5†	430	"	17,000	—	143	270	1.6	1.2
	430	"	17,000	+	151	300	1.8	1.3
6†	650	"	5,600	+	45	51	0.9	0.7
	650	CH ₃ (CH ₂) ₆ C ¹⁴ OONa	6,700	+	50	13	0.2	0.2
7†	400	"	6,700	—	60	46	0.7	0.3
	400	C ¹⁴ H ₃ COONa	5,600	—	60	91	1.6	1.2
8†	485	"	5,600	+	73	80	1.4	1.1
	485	" + oxalacetate	5,600	+	75	80	1.4	1.1
9†	600	CH ₃ C ¹⁴ OONa	8,800	+	48	30	0.3	0.3
	600	" + glucose	8,800	+	50	32	0.3	0.3
	600	" + fructose-6-PO ₄	8,800	+	51	34	0.3	0.3
10†	560	"	8,800	+	80	61	0.7	0.6
	560	" + α-ketoglutarate	8,800	+	83	63	0.7	0.6
	560	" + " + fructose-6-PO ₄	8,800	+	85	65	0.7	0.6

* All experiments designated by the same number were performed on aliquots of pooled liver slices.

† Total recovered radioactivity (c.p.m.)

C.p.m. per μM substrate

‡ Alloxanized.

tomized cats showed a marked reduction in the conversion of acetate to long chain fatty acids, which, as in the case of the alloxanized rat, was not remedied by adding insulin to the incubating medium.

That the failure of conversion of acetate or octanoate to long chain fatty acids is not primarily due to an alteration of tissue permeability to acetate or octanoate in diabetic liver slices was determined by analyzing the acetoacetate formed during these same experiments. A relatively large incorporation of isotope into acetoacetate was obtained with both carboxyl-labeled acetate and octanoate. The slices (Experiment 14, Table II) were able to convert 31 μ M of acetate or 7 μ M of octanoate into aceto-

TABLE II

Conversion of Acetate and Octanoate to Long Chain Fatty Acids by Liver Slices from Normal and Diabetic Cats

Ex- per- iment No.*	Condition of cat	Urinary glucose	Substrate		Insulin	Recovered long chain fatty acids			
			Formula	Radio- activity admin- istered		Per 2.5 gm. liver slices	Radio- activ- ity	Per cent administered radioactivity	Substrate incorpo- rated†
		gm. per kg. per day		c.p.m.		mg.	c.p.m.		μm
11	Fed, normal		C ¹⁴ H ₃ COONa	17,000	—	100	910	5.4	4.0
	" "		"	17,000	+	100	1500	8.8	6.5
12	Fasted normal		"	17,000	—	200	910	5.4	4.0
	" "		"	17,000	+	200	1400	8.2	6.1
13	" diabetic	3.3	"	17,000	—	57	87	0.5	0.4
	" "	3.3	"	17,000	+	61	88	0.5	0.4
14	" "	6.6	CH ₃ C ¹⁴ OONa	12,000	—	180	68	0.6	0.4
	" "	6.6	"	12,000	+	180	68	0.6	0.4
	" "	6.6	CH ₃ (CH ₂) ₆ C ¹⁴ OONa	6,700	—	170	26	0.4	0.2
	" "	6.6	"	6,700	+	175	27	0.4	0.2

* All experiments designated by the same number were performed on aliquots of pooled liver slices.

† See foot-note to Table I.

acetate. Carboxyl-labeled palmitate is also oxidized to acetoacetate by liver slices from alloxanized rats (2 μ M were converted under similar conditions).

Liver slices from alloxanized, as well as from normal rats, readily converted methyl-labeled acetate to cholesterol (Table III). Similarly, liver slices from both normal and pancreatectomized cats utilized acetate for cholesterol synthesis. In five experiments in which pooled liver slices from normal rats were used, the addition of amorphous insulin to the

incubating medium increased significantly the conversion of acetate to cholesterol. With liver slices from alloxanized rats, however, the effect of insulin on this conversion was inconstant.

A few experiments were performed to determine whether prolonged severe "diabetes" would influence the ability of the liver to synthesize cholesterol from acetate. In such cases there was observed a significant reduction in the conversion of acetate to cholesterol.

TABLE III
Conversion of Methyl-Labeled Acetate to Cholesterol by Liver Slices

Animal	Condition	No. of experiments	Insulin	Recovered cholesterol	
				Per cent administered radioactivity	Dilution*
Rat	Normal	7	—	1.5- 8.6	2.7- 9.7
	"	5	+	2.2-12.0	4.0-14.0
	Alloxanized	5	—	1.6-11.0	3.5-14.4
	"	5	+	2.7-11.0	5.3-13.6
Cat	Normal	1	—	2.5	1.9
	"	1	+	4.5	3.2
	Pancreatectomized	1	—	4.5	5.2
	"	1	+	6.2	7.1

Radioactivity of $C^{14}H_2COONa$ = 9600 c.p.m. per mg. of C; 80 μ M of acetate per experiment.

* In per cent of specific activity of acetate.

DISCUSSION

The failure of liver slices from diabetic animals to synthesize long chain fatty acids from acetate or octanoate cannot be ascribed to an inability to form metabolically active 2-carbon fragments from the above substances. The results indicate that the livers of these diabetic animals are quite capable of converting acetate to cholesterol, and in addition, acetate, octanoate, or palmitate to acetoacetate (presumably by way of active 2-carbon intermediates). There is an abundance of evidence that diabetic livers can rapidly oxidize fatty acids to ketone bodies (11). It has been well established (12-15) that, in liver, ketone body formation from octanoate occurs by cleavage into 2-carbon fragments which subsequently are recombined to form acetoacetate. Similarly, it has recently been demonstrated (6) that normal liver slices can transform octanoate into long chain fatty acids by way of intermediate 2-carbon fragments. The diabetic liver is therefore deficient neither in fatty acid oxidases nor in acetoacetate- or cholesterol-forming enzymes.

If it is accepted (1) that acetate and octanoate can be converted into metabolically active 2-carbon fragments by liver slices of diabetic animals and (2) that the fat-oxidizing enzymes (for acetoacetate formation) are unimpaired in such slices, it seems reasonable to suppose, since these liver slices are relatively incapable of synthesizing long chain fatty acids, that the enzymes necessary for the synthesis of such fatty acids from 2-carbon fragments constitute a separate enzyme system. The synthesis of long chain fatty acids is therefore different and distinct from the process of fatty acid catabolism (Fig. 1). This concept would be valid even if there were only one irreversible step involved in fatty acid breakdown.

The fatty acid-synthesizing enzymes may accordingly be relatively non-functional in livers of diabetic animals because of (1) diminished con-

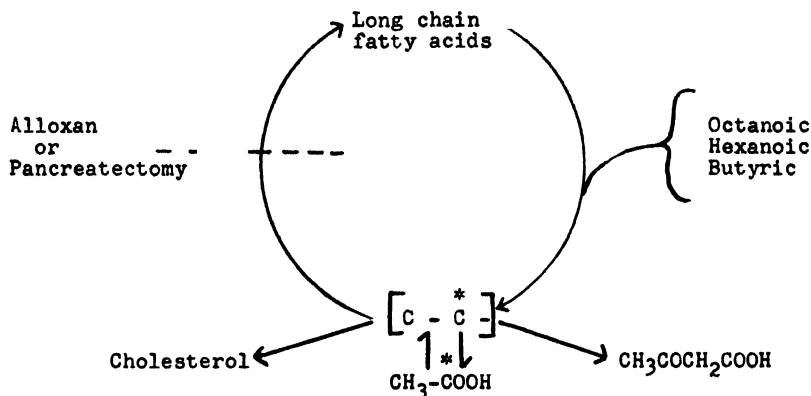


Fig. 1. Catabolic and anabolic metabolism of fat in liver

centration resulting from the inability of diabetic animals to synthesize this enzyme system at an adequate rate, (2) a deficiency of some coenzyme, (3) diminished phosphorylation and non-availability of the appropriate energy-producing reactions, and (4) some indefinite impairment of membrane permeability.

It appears likely that considerable energy is available to the diabetic liver which is capable of actively producing ketone bodies and synthesizing cholesterol. Furthermore, since this process of ketogenesis from fat is oxidative, it would appear unlikely that there is a deficiency of energy-producing reactions in such livers. Moreover, we have found that addition of oxalacetate or α -ketoglutarate does not restore the ability of the diabetic liver to synthesize fat from acetate or octanoate.

It must be admitted, however, that there is an abundance of evidence supporting the thesis that normal fat synthesis occurs only when there is normal carbohydrate metabolism. It is therefore possible that the trans-

fer of energy required for fat synthesis is derived mainly from a coupled reaction involving the simultaneous oxidation of some normal carbohydrate intermediate. Neither glucose and insulin nor fructose-6-phosphate with or without insulin is capable of supplying this hypothetical energy-coupling reaction *in vitro*.

Since relatively large amounts of acetate, octanoate, and palmitate are converted by diabetic liver slices to acetoacetate, it is doubtful that impaired membrane permeability plays any significant rôle so far as these substrates are concerned.

The failure of insulin to reverse this inability to synthesize fat can, for the time being, be attributed only to a deficiency of fat-synthesizing enzymes, to a failure of transfer of energy derived from some normal oxidative step of carbohydrate metabolism, or to impermeability of the "diabetic" liver slice to insulin. It is of interest that Villee and Hastings (16) observed a decreased oxidation of acetate in muscle of alloxanized rats compared with muscle of normal rats. Furthermore, these investigators were unable to reverse this decreased utilization of acetate with insulin.

In any event, the accumulation of fat in the livers of diabetic animals appears to be a result of increased mobilization and transport from extrahepatic sources and not of increased synthesis by the liver. The ability of the diabetic liver slice to synthesize cholesterol from acetate is consistent with the hypercholesterolemia associated with human diabetes. In the absence of fat synthesis from 2-carbon fragments, these fragments must accumulate or be diverted into other metabolic pathways. It is reasonable to assume that this may take the form of increased cholesterol formation and ketone body production.

SUMMARY

Although liver slices from normal rats and cats readily convert labeled acetate to long chain fatty acids, the livers of alloxanized rats and pancreatectomized cats are relatively incapable of carrying out this process. On the other hand, the conversion of acetate to cholesterol by livers of alloxanized rats and pancreatectomized cats is apparently unimpaired, although this process may be inhibited when the diabetes is sufficiently prolonged and severe.

The addition of insulin, glucose, fructose-6-phosphate, oxalacetate, or α -ketoglutarate to the incubating medium fails to reestablish fatty acid synthesis in liver slices from diabetic animals.

The accumulation of liver fat in diabetic animals may be attributed mainly to increased transport from extrahepatic sources.

It is proposed that the synthesis of fatty acids in the liver proceeds by a process independent of fatty acid breakdown.

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RELATION OF TRYPTOPHAN AND NIACIN TO PYRIDINE NUCLEOTIDES OF TISSUE*

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In a previous report (1) from this laboratory it was observed that a tryptophan deficiency in the rat brought about a decrease in activity of several liver enzyme systems. In particular the endogenous respiration of liver tissue was markedly decreased during a tryptophan deficiency. In a similar study of a methionine deficiency (2) it was noted that liver endogenous respiration was not significantly altered. Therefore, it appeared that the effect of the tryptophan upon liver endogenous respiration was unique to that amino acid.

Because of the well known transformation of tryptophan to niacin in animals (3, 4) and since niacin is believed to be the precursor of the liver pyridine nucleotides (DPN and TPN),¹ it was possible that the decrease in liver endogenous respiration induced by a tryptophan deficiency was due to a loss of liver pyridine nucleotides. In the presence of adequate dietary niacin, however, this possibility appeared to be somewhat remote since dietary niacin had been assumed to be capable of maintaining liver pyridine nucleotide content at a normal level even if tryptophan was absent from the ration. The level of niacin in the ration, however, while believed to be adequate under ordinary conditions, might not have been adequate if tryptophan were omitted entirely from the diet.

In the present paper we wish to report some of our findings concerning the interrelationships of tryptophan and niacin in maintaining the pyridine nucleotide content of rat liver. We have investigated the problem both from an analytical and an enzymatic point of view in order to observe the direct involvement of the liver pyridine nucleotides.

EXPERIMENTAL AND RESULTS

Since adult rats had been used in the original study of the effects of a tryptophan deficiency upon tissue enzyme systems (1), the first part of

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¹ DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

this investigation was carried out with adult rats. In order to observe whether still more marked results could be obtained with young growing animals, weanling rats were used in the second part of the experiment.

Adult Animals—Adult male albino rats of the Holtzman strain weighing from 200 to 220 gm. were selected at random from a stock group and divided into six groups of five or six animals each. All the groups were fed a basal ration consisting of the following composition in per cent: acid-hydrolyzed casein (5) 15.29, amino acid mixture² 2.71, niacinless vitamin mixture³ 2, Salts IV (6) 4, corn oil 5, and sucrose to make 100. Three of the groups of animals (I, II, and III) received in addition to the basal ration 0.27 gm. of DL-tryptophan per 100 gm. of basal ration. The remaining three groups (Groups IV, V, and VI) received no tryptophan. Groups I and IV were given no niacin supplement; Groups II and V were given 1.5 mg. of niacin per 100 gm. of basal ration; and Groups III and VI were given 20 mg. of niacin per 100 gm. of basal ration. Thus the experiment was planned so that the effects of a tryptophan deficiency in the absence of dietary niacin, with normal dietary niacin (1.5 mg. per cent), and with high dietary niacin (20 mg. per cent) upon tissue pyridine nucleotide content could be studied. The groups receiving tryptophan and the various levels of niacin served as controls for the corresponding groups receiving no tryptophan.

The animals were maintained on their respective rations until the tryptophan-deficient groups had lost 35 to 40 per cent of their initial body weight. This usually required about 5 weeks from the beginning of the feeding periods.

Since the observation had been made in the earlier study (1) that liver endogenous respiration decreased considerably during a tryptophan deficiency, an attempt was made as a part of the present experiment to remedy the low endogenous respiration by addition of one of the pyridine nucleotides (DPN) to the liver homogenates. If addition of DPN increased endogenous respiration of the livers from the tryptophan-deficient animals without affecting the respiration of the control animals, these results would

² The acid-hydrolyzed casein was assayed microbiologically for the ten essential amino acids and glutamic acid and tyrosine. Those found to be partially destroyed during hydrolysis were repleted with purified amino acids. 2.71 gm. of this mixture contained the following amounts of amino acids: DL-phenylalanine 0.20, DL-methionine 0.16, L-lysine hydrochloride 0.28, L-arginine hydrochloride 0.13, L-leucine 0.11, DL-threonine 0.21, L-histidine hydrochloride 0.25, L-glutamic acid 1.06, and L-tyrosine 0.31. The acid-hydrolyzed casein preparation was found to be completely devoid of both peptides and tryptophan.

³ 100 gm. of vitamin mixture contained the following vitamins in a sucrose base: thiamine hydrochloride 10 mg., riboflavin 15 mg., pyridoxine hydrochloride 12.5 mg., calcium pantothenate 100 mg., biotin 0.5 mg., folic acid 1 mg., choline chloride 5 gm., and *D*-inositol 0.5 gm. Note the absence of niacin.

indicate that the tryptophan deficiency induced a reduction in endogenous respiration by decreasing the liver pyridine nucleotide content. To implement the enzymatic results thus obtained, the livers from the various groups of rats were also analyzed for their content of pyridine nucleotides.

At the end of the feeding periods the animals were sacrificed by decapitation, the livers removed, and divided into two portions. One portion was placed immediately into a dry ice-acetone mixture and analyzed for total liver pyridine nucleotides by the method of Feigelson, Williams, and Elvehjem (7). The other portion was chilled in cracked ice, blotted free of moisture, and homogenized in 5 parts of ice-cold 0.039 M sodium potassium phosphate buffer (pH 7.3) (8). 1 ml. of the resulting homogenate was rapidly pipetted into prepared Warburg flasks kept in cracked ice.

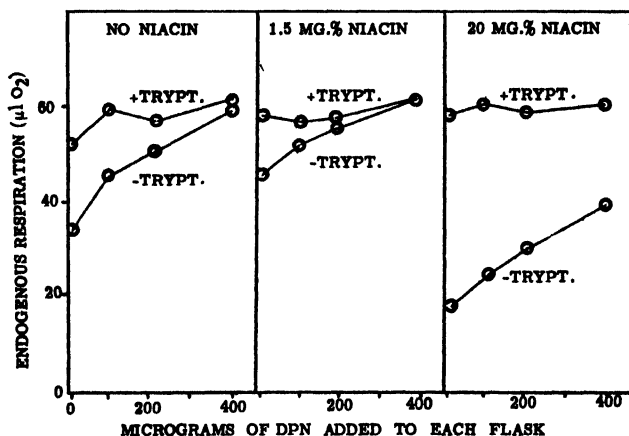


FIG. 1. The effect of DPN added *in vitro* to liver homogenates of normal and tryptophan-deficient rats fed various levels of niacin.

The flasks contained 0.2 ml. of 10 per cent potassium hydroxide in the center well and the following levels of diphosphopyridine nucleotide (DPN), pH 7.2, in the side arms: 0, 100 γ , 200 γ , and 400 γ . Water was added to each side arm to bring the final side arm volume to 0.5 ml. All experiments were run in duplicate. The flasks were equilibrated on a Warburg bath at 30° for 5 minutes, the contents of the side arms added, the stop-cocks closed, and oxygen uptake readings taken at the end of 10 minutes. In this way the quantitative effect of added DPN upon the initial endogenous respiration of the liver was obtained.

The results of the effect of added DPN upon the initial liver endogenous respiration are presented in Fig. 1. The first 10 minute oxygen uptake is plotted against the levels of DPN added to the flasks. The results from the animals of each group were averaged to give the final curves shown

in Fig. 1. In each case the top curve was obtained from animals given adequate dietary tryptophan, while the lower curve was obtained from the groups fed the tryptophan-free rations. The levels of niacin fed the different groups of animals are given in Fig. 1. From the results it can be seen that in the presence of adequate dietary tryptophan the effect of added DPN upon initial endogenous respiration of the liver is insignificant, except perhaps in the animals receiving no dietary niacin. Even in this case the stimulation by DPN is slight. In the absence of dietary tryptophan, however, the stimulation of liver endogenous respiration by added DPN is very marked at all of the levels of dietary niacin. The large decrease in endogenous respiration of the tryptophan-deficient animals receiving 20 mg. per cent of niacin appears to be due to a toxicity of the high level of dietary niacin. In this case the stimulation by added DPN gives

TABLE I
Effect of Dietary Tryptophan and Various Levels of Dietary Niacin on Liver Pyridine Nucleotide of Adult Rats

Group No.	No. of rats	Dietary tryptophan	Dietary niacin	Liver pyridine nucleotides	Range
		<i>per cent</i>	<i>mg. per cent</i>	<i>γ per gm. liver</i>	
I	5	0.27	0	877	820- 942
II	6	0.27	1.5	940	914-1030
III	6	0.27	20	1010	913-1133
IV	6	0	0	465	372- 522
V	5	0	1.5	702	585- 840
VI	5	0	20	699	605- 793

a curve with approximately the same slope as with the other groups of tryptophan-deficient animals, although the lower curve does not reach the normal endogenous respiration level even at the highest level of DPN employed (400 γ).

In Table I are given the analytical results for the concentrations of the pyridine nucleotides found in the livers of the different groups of animals. The total liver pyridine nucleotides, *i.e.* both DPN and TPN, were measured by the method employed for analysis of rat liver (7). However, the analytical results should correlate fairly well with the enzymatic results presented in Fig. 1, since the concentration of DPN in rat liver is much higher than of TPN (9). The results in Table I are reported as the average liver pyridine nucleotide content in micrograms per gm. of fresh liver for the animals of each group. In the last column the range of values for each of the groups is given. From the results it appears that in the absence of both tryptophan and niacin (Group IV) the liver pyridine nucleotide content falls to a very low level, which is brought almost to normal

by feeding tryptophan alone (Group I). If niacin is fed at a 1.5 mg. per cent level without tryptophan (Group V), the liver pyridine nucleotides are increased somewhat over the level observed in the absence of both tryptophan and niacin (Group IV). However, feeding 1.5 mg. per cent of niacin does not bring the liver pyridine nucleotides up to normal unless dietary tryptophan is present (Group II). If a still higher level of niacin is fed, no further increase in liver pyridine nucleotides occurs if dietary tryptophan is absent (Group VI). However, if tryptophan is fed with 20 mg. per cent of niacin (Group III), the liver pyridine nucleotide content is increased to about the same level as that in the rats fed both tryptophan and 1.5 mg. per cent of niacin (Group II). Therefore, from the results it appears that unless rats receive tryptophan the liver pyridine nucleotide content is less than normal, falling very low if both niacin and tryptophan are omitted. However, increasing the niacin to a high level in the ration does not increase the liver pyridine nucleotides to normal when tryptophan is absent. Also, if tryptophan is present, higher levels of niacin have little effect upon liver pyridine nucleotide content.

When the analytical results in Table I are compared with the enzymatic effects of added DPN (Fig. 1), the two types of results agree fairly well. Whenever the liver pyridine nucleotide content is low, addition of DPN to the liver homogenate increases endogenous respiration. When the liver pyridine nucleotide content is normal or almost normal, added DPN has little or no effect upon endogenous respiration. A very striking anomaly occurs in the enzymatic results when 20 mg. per cent of niacin are fed without tryptophan. The marked decrease in endogenous respiration of this group, when compared to the other tryptophan-deficient groups, indicates that excess dietary niacin depresses liver endogenous respiration unless tryptophan is present in the diet. However, the analytical results do not show as low a level of pyridine nucleotides in the liver of this group as the enzymatic results indicate. It is possible that high dietary niacin depresses liver endogenous respiration by a competitive inhibition of pyridine nucleotide utilization.

Weanling Rats—In young growing rats dietary amino acids and vitamins are probably more metabolically limiting than in adult animals, thus permitting the induction of a more complete tryptophan deficiency than possible with adult animals. Therefore, in this part of the experiment weanling rats were used in order to observe whether still more marked effects of tryptophan and niacin upon liver pyridine nucleotides could be demonstrated.

Weanling male albino rats of the Holtzman strain weighing 40 to 45 gm. were divided into four groups of four animals each. The same basal ration fed the adult animals in the first part of the experiment was fed the

weanling animals. Two groups were given the basal ration +0.27 per cent DL-tryptophan (Groups I and II), and the remaining two groups were given the basal ration alone (Groups III and IV). In addition Groups I and III received 5 mg. per cent of niacin, while Groups II and IV received no dietary niacin. The 5 mg. per cent niacin level was chosen in order to insure an excess of dietary niacin over that ordinarily considered adequate for maximum growth (1.5 mg. per cent). The animals were fed the various rations for 2 weeks, at which time the tryptophan-deficient animals had lost 25 to 30 per cent of their original body weight.

The animals were sacrificed by decapitation and the livers removed and analyzed for pyridine nucleotides as previously described (7). The effect of added DPN upon liver endogenous respiration was not measured since the small size of the livers precluded a study of both pyridine nucleotide content and endogenous respiration.

TABLE II
Effect of Dietary Tryptophan and Niacin on Liver Pyridine Nucleotide of Weanling Rats

Group No.	Dietary tryptophan	Dietary niacin	Liver pyridine nucleotides	Range
	<i>per cent</i>	<i>mg. per cent</i>	<i>γ per gm. liver</i>	
I	0.27	5	834	760-925
II	0.27	0	838	820-850
III	0	5	476	437-510
IV	0	0	459	414-544

The results of the liver analyses are presented in Table II. The pyridine nucleotide content of the livers of the different groups of rats is reported as micrograms per gm. of fresh liver. In the last column is given the range of values obtained for each group.

From the results in Table II it appears that the normal level of liver pyridine nucleotides in young rats (Group I) is not quite as high as for normal adult rats (see Table I). If niacin is omitted from the complete ration of young rats (Group II), the pyridine nucleotide content of the liver is unaffected. If tryptophan is omitted, however, and niacin included in the ration (Group III), a marked decrease in liver pyridine nucleotides occurs which is not further significantly decreased by omission of niacin from the ration (Group IV). Therefore, it appears that in young rats dietary tryptophan is more important for synthesis of liver pyridine nucleotides than in adult rats. In young rats dietary niacin appears to have no effect upon liver pyridine nucleotides either in the presence or absence of dietary tryptophan. With adult rats it will be remembered

that, without dietary tryptophan, niacin stimulated synthesis of liver pyridine nucleotides somewhat, although the vitamin was unable to bring liver pyridine nucleotides to normal unless tryptophan was included in the ration.

DISCUSSION

Although dietary niacin is believed to be utilized for synthesis of tissue pyridine nucleotides, our results indicate that tryptophan is much more important than niacin in the formation of these coenzymes. In fact in young growing animals it appears that dietary niacin in the absence of tryptophan does not contribute at all to the synthesis of liver pyridine nucleotides. In the adult rat, however, niacin has been shown to stimulate tissue pyridine nucleotide synthesis in the absence of dietary tryptophan. In these animals inclusion of a normal level of niacin in the ration (1.5 mg. per cent) in the absence of tryptophan increases liver pyridine nucleotides to two-thirds of normal. However, increasing the niacin level beyond 1.5 mg. per cent has no further effect if tryptophan is absent from the ration. Addition of tryptophan to the diet then increases liver pyridine nucleotides to a normal level. Therefore, it appears that in the adult animal one-third of the liver pyridine nucleotides arises from dietary tryptophan regardless of the level of niacin in the ration. In the young growing animal the liver pyridine nucleotides appear to arise completely from tryptophan. An alternative explanation is that dietary tryptophan has a stimulatory effect upon the synthesis of pyridine nucleotides from niacin. In animals fed a niacin-deficient ration some niacin may arise from intestinal synthesis or from the known conversion of tryptophan to niacin. The synthesis of the pyridine nucleotides from niacin could then be facilitated by the remaining tryptophan. The tryptophan-niacin pyridine nucleotide interrelationships uncovered in this study are being further investigated at present.

SUMMARY

1. The effects of dietary tryptophan and niacin in the synthesis of rat liver pyridine nucleotides have been investigated. From the results it appears that dietary tryptophan plays a much more important rôle than dietary niacin in the synthesis of rat liver pyridine nucleotides. In adult rats dietary niacin stimulates synthesis of the pyridine nucleotides although the effect is much less pronounced than that of dietary tryptophan. In young growing rats dietary tryptophan is necessary for the synthesis of the liver pyridine nucleotides, whereas dietary niacin appears to have no effect in maintaining the level of these liver coenzymes.

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XANTHINE OXIDASE AND LIVER NITROGEN VARIATION WITH DIETARY PROTEIN*

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The relation of dietary protein to various enzyme systems in the rat liver has recently been studied by several workers (1-3). As a result of the work in this laboratory, it was observed that one of the liver enzymes most sensitive to variations in dietary protein is xanthine oxidase. Moreover, the activity of this enzyme in the liver was found to be so sensitive to subtle changes in dietary protein that it could be used as an index of amino acid availability in the ration (3).

Further investigations have recently been carried out relating changes in liver enzyme activity to deficiencies of individual amino acids in the rat. Williams, Denton, and Elvehjem (4) have reported the complete disappearance of liver xanthine oxidase activity when rats are deprived of dietary methionine. In subsequent work, Williams and Elvehjem (5) have observed that xanthine oxidase activity of rat liver is much less sensitive to a tryptophan deficiency than to a methionine deficiency. Other observations by Williams, Feigelson, and Elvehjem (6) have indicated that the activity can be decreased to about one-tenth of normal activity by feeding a 6 per cent protein ration. Reports by Bass *et al.* (7) indicated a similar depletion of liver xanthine oxidase by feeding rats an 8 per cent casein diet.

In most of the work mentioned the measurements of liver enzyme activity have been related to general liver protein by basing the changes in activity upon liver nitrogen. It appears that xanthine oxidase activity is lost at a much faster rate than is general liver protein, as shown by a decrease below normal of the QO_2 (N) for xanthine oxidase when rats are fed a deficient protein regimen. In 1936, Addis *et al.* (8) reported that the liver protein of rats fed a non-protein ration is decreased 20 per cent within 2 days of fasting and that the liver is the organ most sensitive to protein deprivation.

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In an attempt to correlate the rate of decrease in activity of liver xanthine oxidase with the decrease in liver protein, we have studied the disappearance of these liver components with time after the animals are placed on a non-protein ration. Although it has previously been shown that the extent of disappearance of liver xanthine oxidase in animals fed a poor protein ration for 2 to 3 weeks is greater than that of general liver protein, the present investigation should reveal the relative rates of disappearance of the two liver components.

It has been reported by Seifter *et al.* (2) and Kensler (9) that low dietary protein induces a decrease in riboflavin concentration in liver. Therefore, the inference may be made that the observed decrease in activity of liver xanthine oxidase is due to a decrease in concentration of liver flavin-adenine dinucleotide (FAD), since FAD is one of the prosthetic groups of xanthine oxidase. We wish to present evidence in this paper that the loss of xanthine oxidase activity in rat liver, induced by a non-protein ration, is due to a loss in enzyme protein *per se* rather than to a loss in the flavin prosthetic group of the enzyme.

EXPERIMENTAL

A total of forty-five male albino rats of the Sprague-Dawley strain, weighing between 175 and 225 gm., was used in these experiments. For a 7 day adjustment period all of the animals were given a ration containing 18 per cent casein + 0.25 per cent DL-methionine before being subdivided into the two groups: a control group, which was continued on the same 18 per cent casein ration, and an experimental group, which was given a non-protein ration.

The basal ration consisted of the following: Smaco casein, 18 gm.; DL-methionine, 0.25 gm.; vitamin mixture (3), 2 gm.; Salts IV (10), 4 gm.; corn oil, 5 gm.; and sucrose to make 100 gm. The protein-free ration was prepared in the same way except that sucrose was substituted for the casein and the methionine. Throughout the experiment, each animal received 2 drops of halibut liver oil, weekly, by dropper.

After the 7 day adjustment period, all but fifteen animals, which served as controls, were placed on the non-protein diet. At this time, three control animals were sacrificed for assay of liver xanthine oxidase. On the following day, five animals of the experimental group were sacrificed for the same purpose. Four experimental animals were sacrificed on the 2nd day for determination of liver xanthine oxidase activity, and, thereafter, groups were sacrificed on the 3rd, 5th, and 7th days. The remaining experimental animals were then returned to the 18 per cent casein + 0.25 per cent DL-methionine ration on the 8th day. 2 consecutive days after this final diet change, assays were made on groups of the experimental

animals to observe the immediate effect of restoration of the dietary protein on liver xanthine oxidase activity, followed by assays on the 14th day, which terminated the experiment. The control animals were sacrificed in groups for determination of liver xanthine oxidase on the 1st, 2nd, 8th, and 14th days of the experiment.

After the prescribed feeding periods, the animals were stunned by a blow on the head, decapitated, and exsanguinated. The livers were removed and placed immediately in cracked ice and chilled for several minutes. They were blotted free of moisture and a portion was weighed and homogenized in 5 volumes of ice-cold 0.039 M sodium phosphate-potassium phosphate buffer (pH 7.4) (11), and xanthine oxidase activity was determined according to the method of Axelrod and Elvehjem (11) with a Warburg bath at 30°.

Total nitrogen was determined on aliquots of the liver homogenates by a modification of the micro-Kjeldahl technique in triplicate.

The results of the xanthine oxidase activity were calculated in terms of activity per gm. of liver fresh weight. The nitrogen value for the homogenates was used for calculating nitrogen per gm. of liver fresh weight.

Another series of experiments was carried out in order to show whether or not FAD concentration in liver is the limiting factor causing a decrease of liver xanthine oxidase activity in rats receiving a non-protein ration. Two groups of male albino rats of the Holtzman strain, weighing 80 to 90 gm., were given, respectively, the 18 per cent casein + 0.25 per cent DL-methionine ration, as previously described, and a non-protein ration in which the casein and methionine were replaced with sucrose. Each group consisted of five animals. The rats were fed their respective rations for 3 weeks, after which they were sacrificed for the enzyme studies.

Since normal rat liver contains approximately 34 γ of riboflavin per gm. of liver (12), it was decided to study the effect of FAD added *in vitro* upon liver xanthine oxidase from levels of 0 to 88 γ per gm. of liver (0 to 25 γ per 284 mg. of liver actually employed in the enzyme determinations). Xanthine oxidase was determined in the liver of each animal by the method of Axelrod and Elvehjem (11). The following levels of FAD were added to duplicate flasks set up for measuring xanthine oxidase activity in the rats receiving the non-protein rations: 0, 1, 3, 5, 10, and 25 γ . For the rats receiving 18 per cent casein and methionine, 0, 1, 2, 3, 5, and 15 γ of FAD per 284 mg* of liver were added.

The FAD was prepared by a method of Huennekens, Sanadi, and Green.¹ The preparation assayed 386 γ per ml. on spectrophotometrical analysis at 450 m μ . The standard D-amino acid oxidase assay indicated 250 γ of

¹ Huennekens, F. M., Sanadi, D. R., and Green, D. E., unpublished method.

biologically active FAD per ml. of preparation. Concentrations of FAD given in this report are expressed in terms of the enzyme assay.

RESULTS AND DISCUSSION

The results of the enzyme studies are shown in Fig. 1 and the liver nitrogen values in Fig. 2. Each point on either graph represents an average of data for three to five animals.

A comparison of Figs. 1 and 2 readily indicates that xanthine oxidase activity per gm. of liver fresh weight is more sensitive to dietary protein changes than is the depletion of liver protein itself. It appears then that this enzyme could be readily affected by subtle changes in the ration, whereas the level of liver protein would respond less markedly to such changes. Although it has been reported that riboflavin is stored in the liver somewhat in proportion to dietary protein, we judge that subtle changes in dietary protein will affect the synthesis of the enzyme *per se* before storage of liver riboflavin or liver protein is affected. As Addis *et al.* (8) pointed out in 1936, 20 per cent of the protein stores of rat liver is depleted in a 2 day fasting period, and we find that xanthine oxidase activity is lost to the extent of 75 per cent of the normal in 2 days on a non-protein ration. It should be pointed out that at least a 48 hour period on the protein-free ration appears to be a prerequisite for a noticeable change in the enzyme activity. This factor indicates that enough protein is present in the liver to sustain liver xanthine oxidase activity for this period. Moreover, it appears that methionine is one of the most limiting amino acids in the synthesis of the enzyme, since it has been shown recently that the enzyme is extremely sensitive to a methionine deficiency (4) and that adding small amounts of methionine to normal 18 per cent casein rations improves the activity of the liver enzyme (3). Moreover, liver xanthine oxidase activity does not appear to be as sensitive to a tryptophan deficiency as to a methionine deficiency (5). Therefore, it seems evident that the effects of a total protein deficiency upon liver xanthine oxidase activity approximate the effects of a methionine deficiency, while they are more drastic than the effects of a tryptophan deficiency.

Information regarding the relative rates of disappearance of xanthine oxidase and liver protein may be observed from the data. From Fig. 1 it can be seen that within 48 hours after placing the animals on a non-protein ration a 75 per cent loss of enzyme activity is observed in the animals on the low protein regimen. In 5 days, there is a complete loss of enzyme activity. (The authors have carried out experiments to show that adding single amino acids, such as methionine or tryptophan, to non-protein rations does not spare the rate of decrease of the enzyme activity.) When the animals were returned to the 18 per cent casein diet, a 15 per

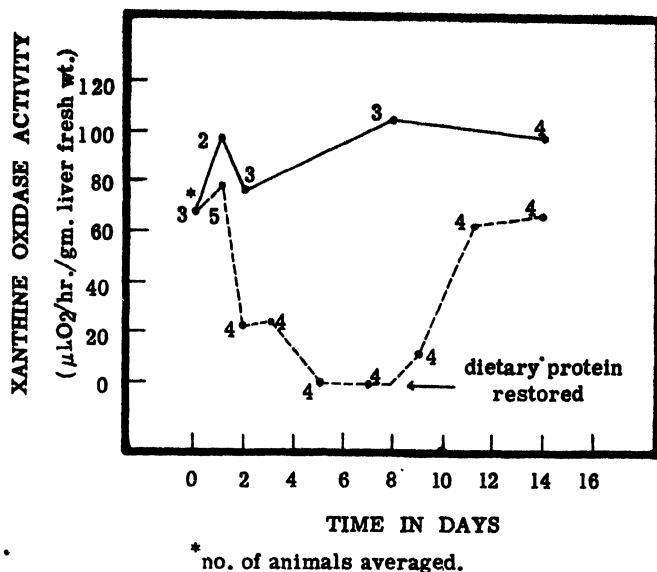


FIG. 1. Variation in activity of liver xanthine oxidase of rats fed a normal ration or a non-protein ration with time after placing the animals on the respective rations. Solid line, rats fed 18 per cent casein + 0.25 per cent DL-methionine ration; broken line, rats fed a non-protein ration.

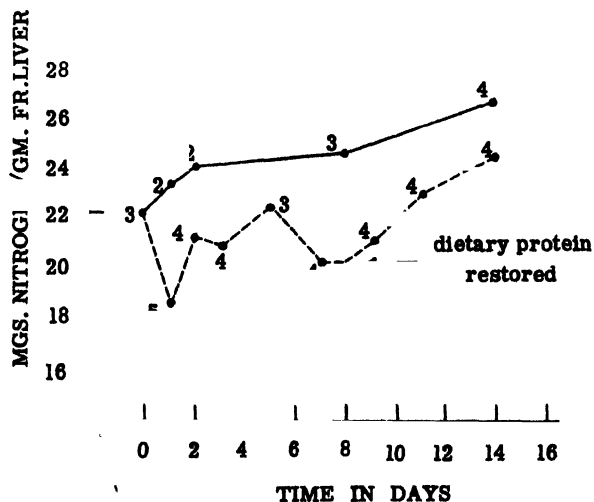


FIG. 2. Variation of liver nitrogen of rats fed a normal ration or a non-protein ration with time after placing the animals on the respective rations. Solid line, rats fed 18 per cent casein + 0.25 per cent DL-methionine; broken line, rats fed a non-protein ration.

cent increase (of normal activity) was noted within 1 day, followed by a 75 per cent increase (of normal activity) on the following day. A steady increase was noted until the experiment terminated.

The liver nitrogen decreased to 80 per cent of normal in 2 days. After the animals were returned to the 18 per cent casein plus 0.25 per cent DL-methionine ration, a steady increase in liver nitrogen was noted, and the normal complement of liver protein was approached as the experiment ended.

TABLE I

Influence of FAD upon Xanthine Oxidase Activity of Liver Homogenates
Normal rats and rats fed a non-protein ration for 3 weeks.

Ration fed	FAD added per 284 mg. liver	Effect on liver xanthine oxidase activity
	γ	$\mu\text{l. O}_2$
18% casein + 0.25% DL-methionine	1	0
	2	-1
	3	-2
	5	+1
	15	0
Average.....		-0.4
Non-protein	1	+3
	3	+4
	5	-2
	10	+2
	25	-4
Average....		+0.6

In Table I are presented the results on the effect of FAD added *in vitro* to the liver homogenates metabolizing xanthine. The figures given denote the actual effect of the added FAD upon oxygen uptake due to oxidation of xanthine. The average xanthine oxidase activity for two groups of animals was approximately the same as for the two groups of animals reported in Fig. 1 at the 7th day of the experiment. When FAD was added to the flasks in which xanthine oxidase activity was being determined, practically no effect was observed either for the rats fed the 18 per cent casein + 0.25 per cent DL-methionine ration or the non-protein ration. The low xanthine oxidase activity encountered in the rats receiving the non-protein ration is no more affected by added FAD than the activity of normal animals. Consequently, it may be concluded from these experiments that the decrease in liver xanthine oxidase observed in rats fed a low

or non-protein ration is not due to a deficiency of FAD because of loss of liver riboflavin but is more probably due to an actual loss in enzyme protein.

SUMMARY

1. Results are presented which reveal that the activity of rat liver xanthine oxidase decreases to 25 per cent of normal after the rats are fed a non-protein ration for 48 hours. Within 5 days of feeding a non-protein ration to the rats, liver xanthine oxidase activity decreases to an immeasurably small amount.

2. Liver nitrogen follows the same trend but is much less sensitive than liver xanthine oxidase activity to the effect of a non-protein ration.

3. The return of liver xanthine oxidase activity to normal is remarkably fast after restoration of dietary protein to animals fed a non-protein ration.

4. Experiments have been reported which indicate that loss in liver FAD appears not to be the cause of the marked decrease in liver xanthine oxidase activity of animals receiving non-protein rations.

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UTILIZATION OF METHIONINE DERIVATIVES BY A MUTANT STRAIN OF *ESCHERICHIA COLI**

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In previous reports, data have been presented concerning the growth-promoting effect of derivatives of certain amino acids (phenylalanine, tyrosine (1), leucine (2), or proline (3)) on artificially produced mutants of *Escherichia coli* (4) which require one of these amino acids for growth. The present communication deals with the response of a *methionineless* strain of *E. coli* (strain 58-161) to derivatives of methionine. This organism is a double mutant derived from the wild type strain K-12; in addition to methionine, biotin is also essential for growth. An earlier study (5) showed that strain 58-161 can grow in a minimal medium supplemented with biotin if one of the following compounds is present: L-methionine, D-methionine, DL-homocystine, DL-homocysteine, L-cystathionine, or D-allo-cystathionine. In its ability to use D-methionine in place of the L isomer, the *methionineless* mutant behaved differently from the *phenylalanineless*, *tyrosineless*, and *leucineless* mutants, for which the D isomers of the respective amino acids did not serve as growth factors (1, 2). It appeared desirable, therefore, to examine more closely the response of strain 58-161 to the stereoisomers of methionine.

It will be noted from Fig. 1 that, if the growth of the *methionineless* mutant is studied as a function of time, the extent of growth ultimately attained with equimolar quantities of D- or of L-methionine is nearly the same; under the experimental conditions employed, however, the cessation of logarithmic growth occurred at about 30 hours in the presence of the L isomer, while growth with the D form was considerably slower and continued until about 45 hours after inoculation. The duration of the lag period is the same in both cases, and it would appear, therefore, that the

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rate of logarithmic growth on D-methionine is limited by some process not involved in the utilization of L-methionine.

When the medium contains both D- and L-methionine, the logarithmic growth response assumes a diphasic character similar to that described by Monod (6), and termed by him "diauxie." The growth curves presented in Fig. 2 show that, when the *methionineless* mutant is provided with the two isomers, it will use the L form preferentially, and then grow at a slower rate with the utilization of the D form. This conclusion is supported by experiments in which D-methionine served as the sole source of

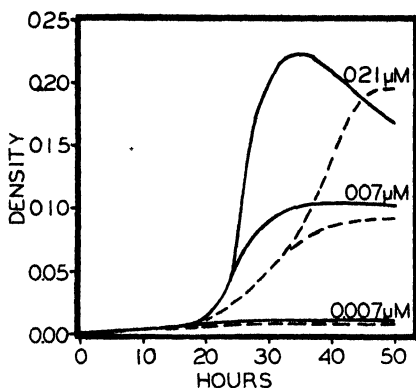


Fig. 1

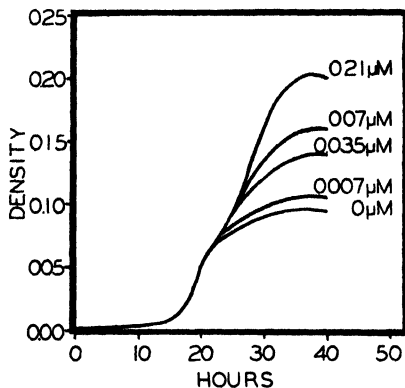


Fig. 2

Fig. 1. Representative growth curves for the *methionineless* mutant in media containing L-methionine (solid lines) and D-methionine (dashed lines). The methionine concentration, in μM per 10 ml., is indicated for each pair of curves.

Fig. 2. Representative growth curves for the *methionineless* mutant in media containing L-methionine plus graded amounts of D-methionine. Each curve represents growth on L-methionine ($0.07 \mu\text{M}$ per 10 ml.) plus the indicated amount, in μM per 10 ml., of D-methionine.

methionine during the first 18 hours of incubation. Upon the addition of L-methionine, the growth response was at once that characteristic for the utilization of this isomer (Fig. 3).

In order to test the possibility that the difference in the growth response to the two isomers might be due to the necessity for the development of adaptive enzymes which would permit the utilization of the D form, the experiments described in Fig. 1 were repeated with inocula obtained from separate cultures grown in the presence of either D- or L-methionine. Since the inocula were taken from actively growing cultures, the lag period was greatly reduced; however, the logarithmic growth response to each of the two isomers was the same for both inocula, and the rate of growth with D-methionine was, in each case, slower than that found with L-methionine.

It does not appear likely, therefore, that the slower growth with D-methionine is due to the development of adaptive enzymes.

Camien and Dunn (7) have reported that, in the presence of sufficient pyridoxamine or pyridoxal, *Lactobacillus arabinosus* 17-5 utilizes D-methionine as well as it does L-methionine, but that, in the absence of these members of the vitamin B₆ group, D-methionine is a poor growth factor. For this reason, the effect of pyridoxal (0.1 to 50 γ per 10 ml.) on the utilization of D-methionine (0.21 μ M per 10 ml.) by strain 58-161 was examined. Identical growth curves were obtained at all concentrations of pyridoxal, and these curves did not differ from the one given in Fig. 1. Under the conditions of these experiments, therefore, the addition of pyridoxal did not enhance the utilization of D-methionine by the *methionineless* mutant.

The fact that strain 58-161 was able to use D-methionine for growth suggested that this organism is capable of deaminating the amino acid to α -keto- γ -methiolbutyric acid, which might then be reaminated to provide the L-methionine required for bacterial growth. It was found that the keto acid, if present in the medium at the time of inoculation, was effective as a growth factor, but that, on a molar basis, 3 times as much of the keto acid was required to produce the extent of growth observed with a given quantity of L-methionine. In view of the extreme instability of the keto acid (8), the possibility was considered that an appreciable amount of it had been destroyed during the lag phase. For this reason, the culture was incubated with a suboptimal quantity of L-methionine for 18 hours after inoculation, and the keto acid was then introduced. It will be noted from Fig. 4 that, under these conditions, the growth-promoting efficiency of the keto acid is increased appreciably. Of especial importance is the fact that the slope of the logarithmic phase of growth in the presence of the keto acid is the same as that found with L-methionine. This suggests that the rate of amination of the keto acid to L-methionine is not a limiting factor in the growth of the *methionineless* mutant in the presence of D-methionine. It may be supposed that some other metabolic step, perhaps that involving the deamination of the D isomer, limits the rate of growth. Although evidence was obtained for the ability of an extract of washed and lyophilized cells of strain 58-161 to effect the oxidation of D-methionine (Table I), insufficient data are at hand to permit a conclusion as to the activity of the bacterial D-amino acid oxidase in the living cells.

It was of interest to find that, when graded amounts of D-methionine were added to a medium containing a constant quantity of the keto acid, the D isomer was not utilized for growth unless it was present in a concentration about 300 times greater than that of the keto acid. On the other hand, if D-methionine was added to the medium after growth on a sub-optimal amount of keto acid had been completed, the D isomer was readily

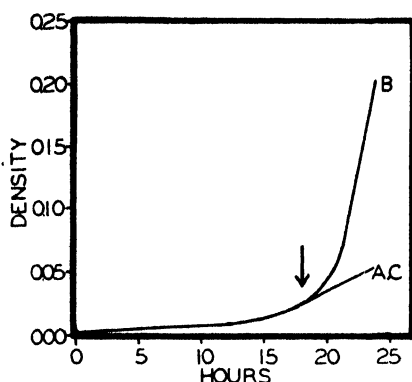


FIG. 3

FIG. 3. Effect of delayed addition of D- or L-methionine to a culture of the *methionineless* mutant. Curve A, $0.07 \mu\text{M}$ of D-methionine, added at 0 hour; Curve B, $0.07 \mu\text{M}$ of D-methionine, added at 0 hour, plus $0.20 \mu\text{M}$ of L-methionine, added at 18 hours (cf. the arrow); Curve C, $0.07 \mu\text{M}$ of D-methionine, added at 0 hour, plus $0.20 \mu\text{M}$ of D-methionine, added at 18 hours.

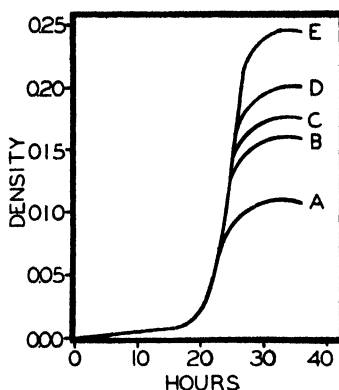


FIG. 4

FIG. 4. Effect of delayed addition of α -keto- γ -methiolbutyric acid on the growth of the *methionineless* mutant. Curve A, $0.21 \mu\text{M}$ of keto acid, added at 0 hour; Curve B, $0.21 \mu\text{M}$ of keto acid, added at 0 hour, plus $0.07 \mu\text{M}$ of L-methionine, added at 18 hours; Curve C, $0.21 \mu\text{M}$ of keto acid, added at 0 hour, plus $0.07 \mu\text{M}$ of L-methionine, added at 0 hour; Curve D, $0.07 \mu\text{M}$ of L-methionine, added at 0 hour, plus $0.21 \mu\text{M}$ of keto acid, added at 18 hours; Curve E, $0.28 \mu\text{M}$ of L-methionine, added at 0 hour.

TABLE I

D-Amino Acid Oxidase Activity of Extract of Methionineless Mutant

The oxygen uptake was measured manometrically in Warburg vessels in the usual manner. Gas phase, air; temperature, 25° ; pH 7.8 (phosphate buffer). The center well contained 0.2 ml. of 5 N KOH; the main chamber contained 1.5 ml. of the cell-free extract (0.3 mg. of protein N per ml.) of strain 58-161, prepared as described in the experimental section; the substrate solutions ($100 \mu\text{M}$ per 0.5 ml.) were placed in the side bulb.

Substrate	Oxygen uptake <i>c.mm. O₂ per hr. per mg. protein N</i>
None	8
L-Methionine	20
D-Methionine	123
" + α -keto- γ -methiolbutyric acid*	17

* $100 \mu\text{M}$ of each substrate were present in the side bulb.

used for growth in concentrations previously found to be effective in the absence of the keto acid. The suggestion may be offered that the keto

acid acts as an inhibitor in the deamination of D-methionine; this is supported by the finding that the oxidation of the D-amino acid is markedly inhibited by the keto acid (Table I). The utilization of L-methionine for growth is not inhibited by the presence of the keto acid in the medium.

When a given quantity of L-methionine is replaced in the medium by an equimolar amount of L-methionylglycine, glycyl-L-methionine, L-methionylglycylglycine, or glycyl-L-methionylglycine, identical growth responses were observed, both with regard to the extent of growth attained and the form of the growth curve. As was to be expected, L-methionyl-L-methionine is twice as active, on a molar basis, as is L-methionine. The utilization of L-methionyl-L-tyrosine was slightly less efficient than that of the other L-methionine peptides tested. Similar results have been noted with other

TABLE II
Effect of Methionine Derivatives on Growth of Methionineless Mutant

Compound	Quantity required for half maximal growth in 30 hrs.
	μM per 10 ml.
L-Methionine	0.075
L-Methionylglycine	0.085
D,L-Methionylglycine	0.14
Glycyl-L-methionine	0.085
L-Methionylglycylglycine	0.075
Glycyl-L-methionylglycine	0.075
L-Methionyl-L-methionine	0.041
L-Methionyl-L-tyrosine	0.095
L-Methioninamide	0.55

mutants of *E. coli*; their growth response to tyrosine-containing peptides was also somewhat less than to comparable peptides in which the tyrosine residue had been replaced by a glycine residue. A comparison of the growth-promoting activity of the L-methionine peptides tested is presented in Table II, which lists the amounts required for the attainment of half maximal growth of the *methionineless* mutant in 30 hours.

Since the *methionineless* mutant is exceptional in its ability to use D-methionine for growth, it was of interest to examine the response of this strain to peptides of the D-amino acid. With D-methionylglycine, growth occurred only with relatively high concentrations of the test substance; to attain the extent of growth achieved in the presence of $0.21 \mu\text{M}$ of L-methionylglycine, the presence of $7.0 \mu\text{M}$ of the D peptide was required. At this level of D-methionylglycine, the slope of the logarithmic phase of growth was similar to that noted in the presence of D-methionine. Glycyl-D-methionine is even less effective as a growth factor than is D-methionyl-

glycine; even with 21 μM of the glycyl peptide, the extent of growth corresponded only to that attained in the presence of 0.07 μM of D-methionine.

Previous studies with other mutants of *E. coli* showed that, when an essential amino acid was replaced in the medium by the corresponding amino acid amide, the amount required for half maximal growth was greatly increased (1, 2, 9, 10), and that the slope of the logarithmic phase depends on the concentration of the amide. A similar result was noted with L-methioninamide as a growth factor for the *methionineless* mutant. In the promotion of half maximal growth in 30 hours, this compound had only 15 per cent of the activity of L-methionine. D-Methioninamide showed even less growth-promoting activity and was comparable to D-methionylglycine in this respect. Of the methionine derivatives tested, glycyl-L-methioninamide was least effective as a growth factor for the *methionineless* mutant, but it exhibited a slight sparing action on the utilization of L-methionine.

It has been suggested previously (10) that the utilization of the peptides of L-phenylalanine, L-tyrosine, and L-leucine by the appropriate mutants of *E. coli* involves initial hydrolysis by bacterial peptidases to yield the essential amino acid, which is then utilized for growth. The data presented above for the *methionineless* mutant are in accord with this view. A preliminary examination of the peptidase activity of saline extracts of lyophilized preparations of strain 58-161 showed the presence of enzymes which, in the presence of added 0.001 M MnSO_4 , caused the hydrolysis of glycyl-L-methionine and of L-methionylglycine at pH 7.9 (0.05 M veronal buffer). At the enzyme concentrations employed (0.02 mg. of protein N per ml. of test solution), no appreciable hydrolysis of glycyl-D-methionine, D-methionylglycine, L-methioninamide, or D-methioninamide could be demonstrated.

EXPERIMENTAL

Measurement of Growth Rate—The procedures employed were essentially the same as those described previously (9). Growth was measured turbidimetrically by means of the Evelyn photoelectric colorimeter (No. 540 filter). The biotin and, in most cases, the test substances were added to the minimal medium (11) before autoclaving. Solutions of D- and L-methioninamide, glycyl-L-methioninamide, pyridoxal, and α -keto- γ -methylolbutyric acid were sterilized by filtration and added to the medium after it had been autoclaved. The concentration of the test substances is given in terms of μM per 10 ml. of medium. An inoculum from a 24 hour-old agar slant was made up to uniform density (0.027) and 0.1 ml. of the bacterial suspension was added to 10 ml. of the medium. The incubation temperature was 30° throughout. During the logarithmic phase, the

extent of growth was determined at 2 hour intervals. To facilitate inspection of the growth curves in Figs. 1 to 4, the experimental points have been omitted.

Cell-Free Extracts—The *methionineless* mutant was grown in 6 liters of minimal medium to which methionine and biotin had been added. At the point of maximal growth the cells were centrifuged, and then washed three times with distilled water. Upon lyophilization, 1 gm. of dried cells was obtained. To prepare a cell-free extract, 150 mg. of the bacteria were ground with alumina (60 mesh) and 4.5 ml. of 0.1 M phosphate buffer (pH 7.9) for 10 minutes. The resulting paste was centrifuged and the supernatant fluid was used for the experiments described in Table I.

Test Substances—The synthesis of all but two of the methionine derivatives used in the present study has been described in a previous report (12).

Carbobenzoxyglycyl-L-methioninehydrazide—2.8 gm. of carbobenzoxyglycyl-L-methionine methyl ester (12) were dissolved in 100 ml. of absolute alcohol, 1.2 ml. of hydrazine hydrate were added, and the reaction mixture was allowed to stand at room temperature. After 4 days, 1.2 gm. of the crystalline product had separated; a second crop of 0.6 gm. was obtained by chilling the filtrate; m.p., 138–139°.

$C_{15}H_{21}O_4N_3S$ (354.4). Calculated, N 15.8; found, N 16.0 (Dumas)

Carbobenzoxyglycyl-L-methionylglycine Benzyl Ester—1.2 gm. of the above hydrazide were dissolved in a mixture of 30 ml. of water, 1.1 ml. of glacial acetic acid, and 0.5 ml. of concentrated hydrochloric acid, and converted to the azide by the addition of 400 mg. of $NaNO_2$ in 10 ml. of water. The azide was extracted with 100 ml. of ethyl acetate, and the ethyl acetate solution was washed with water, aqueous bicarbonate, and water, and then dried over Na_2SO_4 . The azide solution was added to an ethyl acetate solution of glycine benzyl ester (from 1.7 gm. of the hydrochloride) and allowed to stand at room temperature overnight. The reaction mixture was then washed successively with dilute hydrochloric acid, water, aqueous bicarbonate, water, dried over Na_2SO_4 , and concentrated *in vacuo*. The resulting crystalline residue was recrystallized from ethyl acetate-petroleum ether. Yield, 0.7 gm.; m.p., 148–149°.

$C_{24}H_{31}O_6N_3S$ (487.5). Calculated, N 8.6; found, N 8.8

Glycyl-L-methionylglycine—0.7 gm. of carbobenzoxyglycyl-L-methionylglycine benzyl ester was hydrogenated with palladium-black as the catalyst. After removal of the palladium by filtration, the solution was concentrated to a small volume under reduced pressure. The unchanged carbobenzoxy tripeptide ester (0.3 gm.) crystallized and was filtered. The filtrate was concentrated to dryness *in vacuo*, and the residue was recrystallized from

water-ethanol. Yield, 130 mg.; m.p., 222° (with decomposition); $[\alpha]_D^{24} = -30^\circ$ (1.8 per cent in water).

$C_8H_{17}O_4N_2S$	Calculated.	C 41.1, H 6.5, N 16.0
(263.3)	Found.	" 41.3, " 6.5, " 16.3

α -Keto- γ -methiolbutyric Acid—DL-Methionine was treated with swine kidney D-amino acid oxidase (13) and the resulting keto acid was isolated in the form of its 2,4-dinitrophenylhydrazone, which was then converted to the sodium salt of the free keto acid (14).

$C_6H_7O_3SNa$ (170.2). Calculated, S 18.8; found, S 18.3

SUMMARY

A study has been made of the growth-promoting activity of D- and L-methionine and their derivatives for a *methionineless* mutant strain of *Escherichia coli*. A difference has been observed in the growth response of the mutant to D- and L-methionine, and this has been attributed to the presence of a limiting step in the conversion of the D isomer to the L form, via the corresponding α -keto acid. The response of the *methionineless* mutant to peptides of L-methionine is in accord with the view that these peptides undergo prior hydrolysis to yield the essential amino acid, which then serves as a growth factor.

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CALCIUM DETERMINATION BY FLAME PHOTOMETRY; METHODS FOR SERUM, URINE, AND OTHER FLUIDS

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The convenience and accuracy of flame photometric determinations of sodium and potassium in biological fluids (1, 2) have suggested the desirability of extending these advantages to determinations of calcium. Since calcium emission lines are much less intense than those of sodium and potassium (3), the samples of diluted serum (1:50) customarily used for sodium and potassium determinations are not adequate for the measurement of calcium concentrations. Serum dilutions of the order of 1:10 are adequate provided their viscosity is reduced by precipitation of the proteins with trichloroacetic acid. At the wave-length used for calcium measurements, a correction is necessary for the effect of sodium and potassium upon calcium emission and upon flame background. The correction may be easily derived. The resulting determinations of calcium agree well with those obtained by the Van Slyke-Sendroy technique and in our hands are somewhat more rapid and accurate.

Method

Preparation of Serum—1.00 cc. of serum and 5.00 cc. of distilled water were accurately pipetted into 15 cc. conical centrifuge tubes fitted with glass stoppers and mixed. 4.00 cc. of 10 per cent trichloroacetic acid were added, the stopper inserted, and the solution mixed by inverting ten times. 10 minutes were allowed for protein precipitation. The tubes were then centrifuged at 2000 r.p.m. for 10 minutes. About 3 cc. of supernatant were decanted or pipetted into the 5 cc. atomizer beakers.

Urine and Other Fluids—Samples of urine and other fluids for calcium measurements were prepared in a manner similar to that for serum. The final concentration of trichloroacetic acid was 4 per cent and the dilution of the sample was such as to bring the concentration of calcium into the 0.1 to 1.0 m.eq. per liter range. Aliquots of urine and other fluids must be taken from well mixed specimens, since sediments often contain large amounts of calcium. This calcium is brought into solution in 4 per cent trichloroacetic acid (4).

Note—Filter paper should be avoided at all stages in the preparation of

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the samples, since acid solutions will extract a measurable amount of calcium from most "ash free" filter papers (4).

Preparation of Standards—A stock solution of 10.00 m.eq. of calcium per liter was prepared by dissolving 0.5004 gm. of CaCO_3 (Iceland spar) in 10 cc. of concentrated HCl and diluting it to 1 liter with distilled water. The usual working standard made from this solution contained 0.5 m.eq. of calcium, 14.0 m.eq. of sodium, and 0.5 m.eq. of potassium (as chlorides) per liter of 4 per cent trichloroacetic acid solution, corresponding essentially to a 1:10 dilution of serum in 4 per cent trichloroacetic acid. For recovery experiments, a standard with 1.0 m.eq. of calcium per liter was prepared. The reading for the calcium blank was determined with a solution containing 14.0 m.eq. of sodium and 0.5 m.eq. of potassium per liter of 4 per cent trichloroacetic acid.

All preparations of 10 per cent trichloroacetic acid solution we have measured have contained 0.01 to 0.02 m.eq. of calcium per liter. When diluted to 4 per cent solution, the actual concentration is 0.004 to 0.008 m.eq. per liter, which contributes 0.04 to 0.08 m.eq. per liter to the reading of calcium, an error of 0.8 to 1.6 per cent of normal serum concentration. The effect of this contribution is cancelled by including 4 per cent trichloroacetic acid in the standards. Trichloroacetic acid at this concentration has no measurable effect on the flame emission of sodium or calcium.

Instrument—The instrument used was a Beckman quartz spectrophotometer with a flame attachment. Light from the flame was measured on the transmission scale of the instrument, since the units on this scale are related linearly to the intensity of the light. The settings of the instrument are somewhat arbitrary, the following serving only as a guide. Calibration curves may vary somewhat with different settings and different instruments and should be checked for each instrument.

Oxygen—22 inches of water pressure.

Gas—Adjusted to give medium small cones of blue flame, about 0.3 to 0.4 cm. in height. Shorter cones tended to cause an unstable reading. The propane pressure gage read about 1.2 cm. but was not used in flame adjustment.

Air—20 pounds per sq. in.

Wave-Length—The 556 $m\mu$ calcium oxide line is the most satisfactory for calcium measurement.

Sensitivity—Adjusted to give readings on the transmission scale of about 70; about four turns of the sensitivity knob from the counter-clockwise limit with switch position 0.1.

Phototube—The ultraviolet tube was used at 556 $m\mu$; it is more sensitive than the red phototube.

Slit Width—0.5 mm. Wider slits admit a higher proportion of background light. Narrower slits admit less calcium light than is needed for accurate measurement. Slits wider than 1.2 mm. admit the intense 589 $m\mu$ sodium light.

Measurement—The reading on the transmission scale was recorded for distilled water, for the sodium- and potassium-containing calcium blank, for the working standard, and for the unknown, followed by repeated readings of the standard and unknown to determine the average difference between the two readings. After three pairs of agreeing readings were obtained, the calcium blank and distilled water readings were checked. When necessary, data for a sodium and potassium correction were obtained by reading the transmission at 589 $m\mu$ and at 767 $m\mu$ of the standard and unknown solutions. For this purpose the slit was decreased to about 0.2 mm., the sensitivity was increased if necessary to 1.0, and the red photocell was used.

Effect of Sodium and Potassium upon Calcium Measurements at 556 $m\mu$ —Sodium and potassium add to the flame background and also increase the intensity of the basic calcium emission at 556 $m\mu$. Milliequivalents of sodium and potassium are approximately interchangeable in producing both of these effects. The effect on basic calcium emission is plateau-like, being about 9 per cent for sodium plus potassium concentrations between 1.0 and 24.0 m.eq. per liter in the solution being nebulized. Inclusion of sodium or potassium in concentrations greater than 1.0 m.eq. per liter in blanks, standards, and unknown solutions cancels out this plateau-like effect.

The effect upon flame background is progressive, but if the concentrations of sodium and potassium in the unknown and standard solutions are reasonably similar, as in the case of most serum determinations, no correction for background effect is necessary. A sodium variation (from 140 m.eq. per liter) of 10 per cent will introduce no more than 2.5 per cent error in serum calcium determination when the recommended sodium- and potassium-containing blank and standard solutions are used as points of reference. A potassium variation (from 5 m.eq. per liter) of ± 5 m.eq. per liter will introduce no more than 0.5 per cent error. Accurate calcium measurement in solutions other than serum requires that the sodium and potassium background light in the unknown be computed to provide a calcium blank for the unknown. The method requires measurement of sodium and potassium concentration as described below.

Calculation of Sodium Concentration—The sodium concentration of the unknown solution is determined from the 589 $m\mu$ reading by reference to Fig. 1. This curve has been derived by plotting, as fractions of the transmission reading (T) of the standard at 589 $m\mu$, the transmission reading of

a series of solutions of known sodium concentration. From the observed ratio of the T (unknown) to T (standard), the sodium concentration of the unknown solution is read from the graph.

Calculation of Potassium Concentration—The potassium concentration of the unknown is determined from the 767 $m\mu$ readings by reference to Fig. 2. These curves have been derived in a manner similar to that used for sodium concentration, by recording at 767 $m\mu$ the transmission of a series of solutions with known sodium and potassium concentrations. For both Fig. 1 and Fig. 2 the readings of the calcium standard, or calcium blank, which contain 14.0 m.eq. of sodium and 0.5 m.eq. of potassium per liter, are

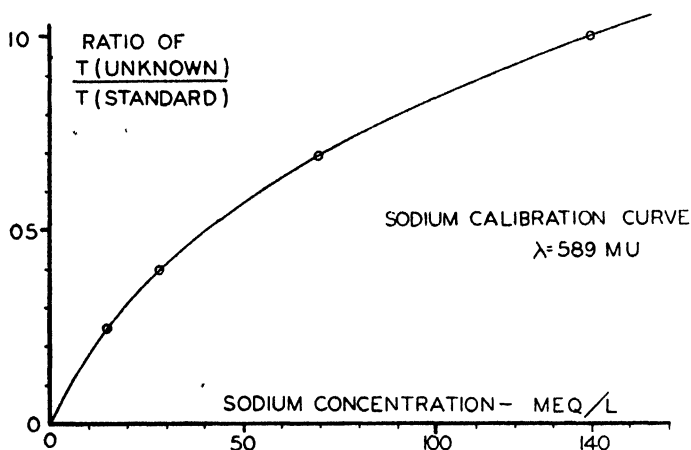


FIG. 1. Sodium calibration curve for determining the concentration of sodium in unknown solutions. The transmission readings of a series of sodium solutions are plotted as ratios in which the transmission reading of a 140 m.eq. per liter standard read in 1:10 dilution is given the value of 1 in the denominator. (Potassium, calcium, and magnesium in concentrations less than 10 times those found in serum have no measurable effect on sodium emission.)

taken as the reference standard and plotted as 1.00. From the previously calculated sodium concentration and the observed ratio of T (unknown) to T (standard), the potassium concentration may be read from the graph.

Computation of Calcium Blank Reading for Unknown—The calcium blank reading for the unknown is the reading of background light due to sodium and potassium in the unknown. Fig. 3 is employed to calculate this reading as a fraction of the standard calcium blank reading. Fig. 3 is obtained by measuring at 556 $m\mu$ solutions containing no calcium but varying amounts of sodium and potassium. Again the transmission reading of the sodium- and potassium-containing calcium blank solution is plotted as 1.00.

Location of the sodium and potassium concentrations of the unknown on these curves indicates a factor on the abscissa. This factor when multiplied by the 556 $m\mu$ reading of the calcium blank gives the corrected calcium blank for the unknown. See Table I for an example of the calculation.

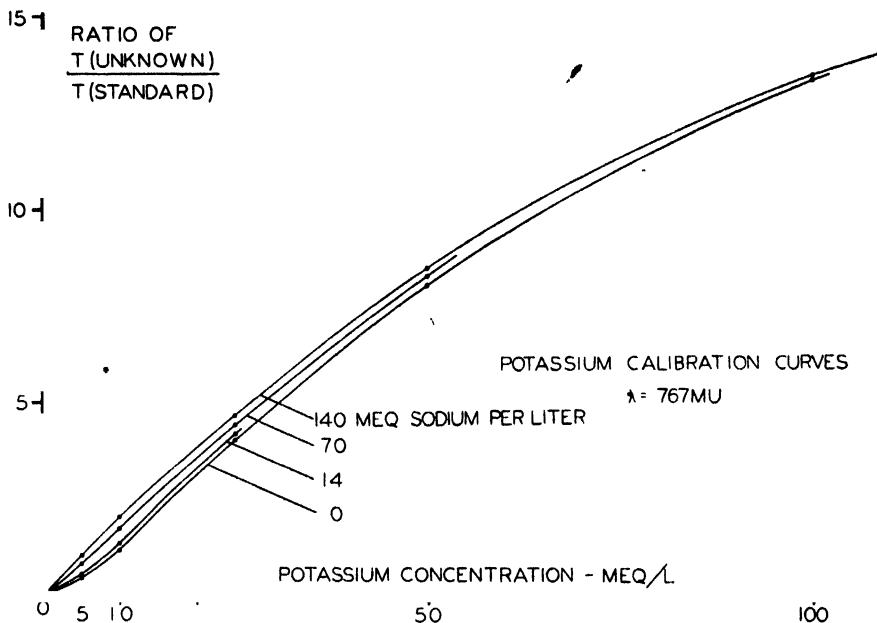


FIG. 2. Potassium calibration curves for determining the concentration of potassium in unknown solutions. The curves for solutions of potassium containing varying amounts of sodium are given, since potassium emission is increased by sodium. In plotting the transmission ratios on the ordinate, the transmission reading of a standard containing 140 m.eq. of sodium and 5 m.eq. of potassium per liter, read in 1:10 dilution, was given the value 1. (Calcium and magnesium in concentrations less than 10 times those found in serum have no measurable effect on potassium emission.)

Calculation of Calcium Concentration—Since calcium light at 556 $m\mu$ is a linear function of calcium concentration (5), an unknown calcium concentration may be measured by employing a single calcium standard and a calcium blank to determine the slope of the line relating concentration and intensity of emitted light. A simple proportion then relates the concentrations and the transmission readings (corrected by subtracting calcium blanks) of the unknown and standard solutions.

$$\frac{\text{Ca (unknown)}}{\text{Ca (standard)}} = \frac{T \text{ (unknown)}}{T \text{ (standard)}}$$

or, with a 1:10 dilution of both the 5.00 m.eq. per liter calcium standard and the unknown solution,

$$\text{Ca (unknown)} = \frac{5.00 \times T \text{ (unknown)}}{T \text{ (standard)}} \text{ m.eq. per liter}$$

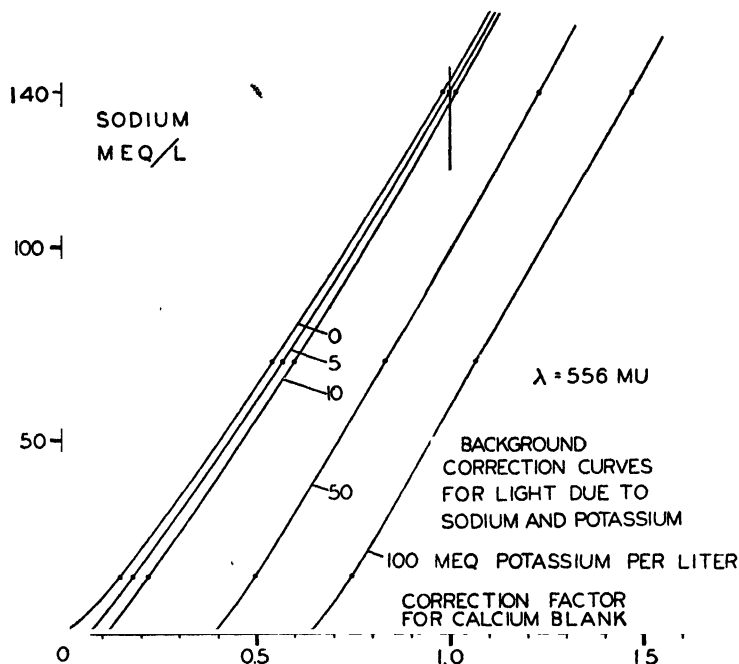


FIG. 3. Background correction curves for determining the appropriate calcium blank for unknown solutions of calcium containing varying amounts of sodium and potassium. The curves are constructed by recording in 1:10 dilution the transmission readings at 556 μ of a series of solutions containing known amounts of sodium and potassium and plotting these readings on the abscissa as ratios in which the reading of the standard calcium blank in the denominator is given the value 1.0.

Example with data given in Table I:

$$\text{Ca (unknown)} = 5.00 \times \frac{(29.1 - 19.4 - 2.3)}{(86.0 - 19.4 - 6.7)} = 5.00 \times \frac{7.4}{39.9} = 0.928 \text{ m.eq. per liter}$$

Determination of Calcium by Van Slyke Gasometric Technique—The modified method of Sendroy was used (4). Calcium was precipitated from 1 cc. of serum plus 5 cc. of water by adding 1 cc. of saturated ammonium oxalate. The solution was mixed, allowed to stand 16 hours, and centrifuged 5 minutes, at 2600 r.p.m. All but 0.2 to 0.3 cc. of supernatant was aspirated with a pipette having an upturned tip. The precipitate was

washed three times with 3 cc. of fresh 2 per cent ammonia water, with use of 1 cc. to wash down the wall and 2 cc. to promote mixing by running it rapidly down the slanted tube wall. Centrifuging and aspirating were done between washes as above. CO_2 was released from the oxalate with ceric sulfate.

TABLE I
Calculation of Corrected Calcium Blank

Reading on transmission scale			
At 556 $m\mu$	At 589 $m\mu$	At 767 $m\mu$	
19.4	0	1.0	Distilled water
26.1	24.0	29.1	Calcium blank (4% trichloroacetic acid containing 14 m.eq. sodium and 0.5 m.eq. potassium per liter)
66.0	24.0	29.1	Calcium standard (4% trichloroacetic acid containing 14 m.eq. sodium, 0.5 m.eq. potassium, and 0.5 m.eq. calcium per liter)
29.1	8.6	91.3	1:10 dilution of unknown urine (and trichloroacetic acid, final concentration 4%)

(1) The ratio of the readings of the unknown solution and the calcium blank at 589 $m\mu$ = $8.6/24.0 = 0.36$. (2) From Fig. 1, the sodium concentration is 25 m.eq. per liter. (3) The ratio of the readings of the unknown solution and the calcium blank at 767 $m\mu$ = $(91.3 - 1.0)/(29.1 - 1.0) = 3.2$. (4) From Fig. 2, the potassium concentration is 17 m.eq. per liter. (5) From Fig. 3, the correction factor for the calcium blank is 0.35. (6) The standard calcium blank at 556 $m\mu$ = $26.1 - 19.4 = 6.7$. The calcium blank of the unknown solution = $6.7 \times 0.35 = 2.34$.

Results

Table II is a record of twenty-three serum calcium measurements before and after addition of known amounts of calcium. The average recovery of about 1 per cent more than was added corresponds well with the expected effect on calcium concentration introduced by the volume displacement incident to protein precipitation.¹

¹ Protein constitutes 6 per cent by weight of normal serum, which is 4.5 per cent by volume, assuming a specific volume of protein of 0.75 (6). In a 1:10 dilution, this volume is 0.45 per cent of the total. If this is all precipitated and centrifuged into the bottom of the tube, this space is no longer available for calcium (and other solutes), which are thereby displaced upward, increasing the supernatant calcium concentration by 0.45 per cent. Experimentally, working with 1:5 dilutions, Sendroy (4) gives the magnitude of this effect as 2.5 per cent, which in 1:10 dilution is 1.25 per cent. The recovery experiments presented in Table II indicated a value of

TABLE II
Recovery of Calcium Added to Serum

Serum No.	Calcium	Plus 4.00 m.eq. per liter	Recovery
	<i>m.eq. per l.</i>		<i>m.eq. per l.</i>
1	4.93	8.98	4.05
1	5.00	9.15	4.15
1	5.03	9.15	4.12
2	4.70	8.65	3.95
3	4.84	8.87	4.03
4	4.97	9.05	4.08
5	4.43	8.50	4.07
8	5.36	9.39	4.03
Average			4.06 = +1.33%
S.d.m., σ			0.057
		Plus 5.00 m.eq. per liter	
6	4.64	9.75	5.11
7	5.86	10.88	5.02
9	5.13	10.15	5.02
10	5.42	10.45	5.03
11	5.36	10.38	5.02
12	4.90	9.93	5.03
13	5.40	10.37	4.97
14	5.23	10.32	5.09
15	4.87	9.88	5.01
15	4.95	9.99	5.04
17	4.74	9.75	5.01
18	4.78	9.83	5.05
18	4.79	9.79	5.00
20	4.68	9.78	5.10
20	4.69	9.76	5.07
Average . . .			5.040 = +0.8%
Combined average re- covery error			+0.97%
S.d.m., σ			0.038

In Table III the data obtained in calcium determination by flame analysis and by gasometry on the same sera are compared. In this series, about 1 per cent. Since these values are about 2 to 3 times the effect predicted by the volume of the protein, it is assumed that the precipitating agent is bound with the protein, increasing the volume of the precipitate. The amount of calcium bound or occluded in the precipitate is not known. Until a more accurate estimate of the magnitude of the volume displacement effect is obtained, a correction of -1 per cent is applied to calcium determinations on protein-free preparations.

the results with the flame photometer have all been lowered 1 per cent to correct for volume displacement. The average of differences between the methods was 0.2 per cent and is not significant.

Many of the sera were measured several times by flame photometry and by gasometry. Gasometric determinations were usually done in du-

TABLE III
Comparison of Results by Gasometry and Flame Photometry

Serum No.	Flame photometer calcium	Gasometric calcium	Difference	
			<i>m.eq. per l.</i>	<i>per cent</i>
18	4.72	4.70	+0.02	+0.4
19	4.61	4.58	+0.03	+0.6
19	4.62	4.64	-0.02	-0.4
19	4.70	4.70	0.00	0.0
21	4.48	4.55	-0.07	-1.4
22	4.81	4.71	+0.10	+2.0
23	4.71	4.72	-0.01	-0.2
23	4.71	4.68	+0.03	+0.6
23	4.73	4.72	+0.01	+0.2
23	4.73	4.72	+0.01	+0.2
24	4.71	4.73	-0.02	-0.4
25	4.83	4.79	+0.04	+0.8
Average			±0.01	+0.2
S.d.			0.04	0.8

All flame photometer determinations were averages of multiple readings on a single serum preparation and have been corrected for volume displacement effect by -1 per cent.

TABLE IV
Reproducibility of Calcium Determinations by Flame Photometry

No. of determinations done on 17 sera measured more than once	50
Maximum difference between any two readings on same serum	0.15 m.eq. per liter
S.d.m.	0.026 m.eq. per liter = 0.5%

plicate or triplicate. Table IV demonstrates the reproducibility of the two methods. The largest difference between duplicates in 50 flame analyses of seventeen sera was 0.15 m.eq. per liter. The standard deviation from the mean was 0.5 per cent. None of the determinations carried out on the flame photometer by the above method was excluded for any reason. In the Van Slyke series, because of the more complicated pro-

cedures, occasional large errors occur and duplicates differing by more than 2 per cent were not included.

DISCUSSION

The results obtained with the above procedures for calcium measurement suggest that flame photometric determinations in our hands are slightly more accurate and reproducible than are gasometric determinations.

The accuracy of the method depends primarily on volumetric technique. The variability of flame measurement may be reduced by repeated readings to no more than 1 per cent.

SUMMARY

Calcium in serum, urine, and other fluids may be rapidly and easily measured by flame photometry. The accuracy of the method is equal to that of the gasometric determination of calcium oxalate. In the case of serum, proteins are precipitated in 4 per cent trichloroacetic acid, and the supernatant solution, effectively a 1:10 dilution of the serum electrolytes, is nebulized into a constant flame. The intensity of the flame light at 556 $m\mu$ is compared with that produced by standard solutions containing calcium, sodium, potassium, and trichloroacetic acid. A correction for the effect of variations in sodium and potassium concentration in unknown solutions of urine and biologic fluids may be made. For serum calcium determinations, the usual stability of serum sodium and potassium concentration makes correction for sodium and potassium variations of limited value.

The writers wish to acknowledge their indebtedness to Mr. John Rowe, Harvard Medical School, for his assistance in developing the above method for calcium determination.

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PHYSICAL STUDIES ON THE ADRENOCORTICOTROPIC HORMONE OF THE SWINE ADENOHYPOPHYSIS*

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Several years ago, it was reported independently from two laboratories that pituitary adrenocorticotrophic hormone (ACTH), isolated from sheep (2) or hog (3) glands, was a protein of relatively low molecular weight, namely, 20,000. The methods of isolation were substantially different; yet both groups of investigators were in agreement concerning the protein nature of the hormone and its main chemical and physical characteristics.

Our investigation was undertaken in an attempt to define with more precision the physical properties of the hormone isolated by the method of Sayers, White, and Long (3). This paper is concerned with the ultracentrifugation, electrophoresis, and diffusion of ACTH. The results are discussed with reference to the nature of the hormone as it exists in the pituitary gland and to recent reports (4) that hormone activity may be associated with moieties which are smaller and more active than the proteins isolated in earlier studies.

EXPERIMENTAL

Materials—ACTH was isolated from lyophilized anterior pituitary lobes of swine by the procedure of Sayers *et al.* (3). During the course of the investigation, four different preparations were made,¹ which had essentially the same biological activity when assayed in hypophysectomized rats by the adrenal ascorbic acid depletion method of Sayers *et al.* (5). These preparations were equal in potency to the Armour standard, La-1-A. In addition, each of these preparations was studied under identical conditions in the ultracentrifuge and Tiselius apparatus. These results will be discussed in detail, but it may be stated here that, since the method of isola-

* This investigation was supported by research grants from the National Institutes of Health, United States Public Health Service, and Armour and Company.

Part of this work was presented at a meeting of the American Society of Biological Chemists in April, 1950 (1).

¹ We wish to acknowledge the help of John B. Richards in the preparation of the hormone.

tion was quite reproducible and gave products which had similar properties, no specific identification of the different preparations will be given.

Electrophoresis—Prior to the realization that activity of ACTH is labile at neutral and slightly alkaline pH values (1), we used as a standard electrophoretic assay of the purity of the hormone the migration of the descending boundary in veronal buffer at pH 8.5 and at an ionic strength of

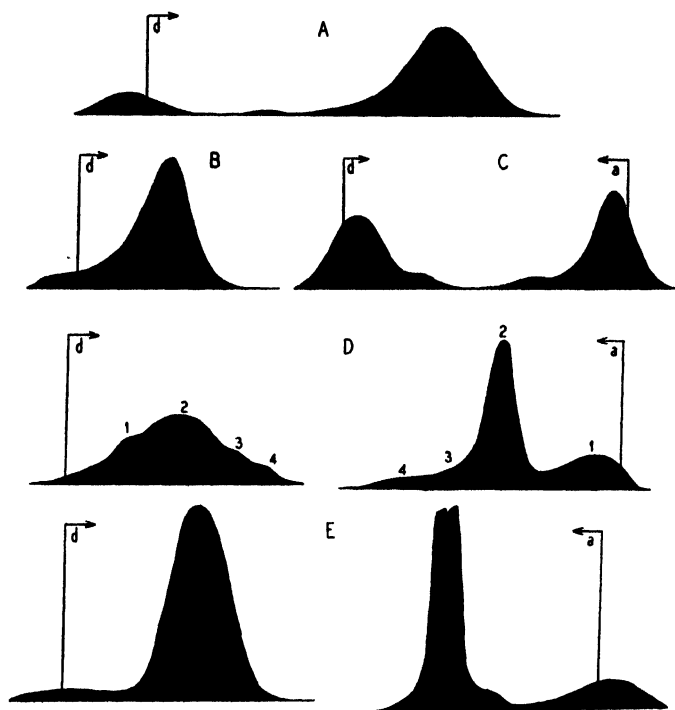


FIG. 1. Electrophoretic patterns of ACTH. *A* is the descending pattern in veronal at pH 8.5 after migration for 250 minutes. *B* is the descending pattern in acetate at pH 5.3 after migration for 200 minutes. *C* shows ascending (*a*) and descending (*d*) patterns in citrate at pH 4.1 after 200 minutes. *D* indicates the results in acetate after 300 minutes. *E* shows the patterns in acetate at pH 2.7 after 200 minutes. All the buffers were at an ionic strength of 0.1. The experiments were performed at a potential gradient of about 5.5 to 6.0 volts per cm.

0.1. The preparations employed in our studies gave a main component which constituted 90 to 94 per cent of the migrating material if one assumes a completely symmetrical boundary; 6 to 10 per cent of the area was represented as a small asymmetry on the slowly moving side of the main component. A typical experiment is shown in Fig. 1, *A*; the main component represented 94 per cent of the total area and there was less than

1 per cent of a slow component in addition to the asymmetry on the slow side of the major peak. It may be noted that the lower edge of the pattern is the base-line, which is quite flat but has a slope which deviates slightly from the horizontal.

Other studies were made of the electrophoretic behavior of ACTH at more acid pH values. Examples of these are also shown in Fig. 1. In acetate at pH 5.3 (Fig. 1, *B*), the appearance was similar to that in veronal. However, in acetate at pH 3.6 and 4.0, values which are on the acid side of the isoelectric point, the behavior was quite different. Three or four boundaries were observed under these conditions (Fig. 1, *D*). The main component represented only 40 to 70 per cent of the total area in several experiments. Similar results were obtained in a single run in propionate at pH 3.8. The polydispersity of the preparations cannot be ascribed entirely to the pH. The protein is exceedingly soluble in acetate buffer, and solutions containing 2.5 or 3 per cent protein were easily prepared. In contrast, it was found that our preparations of ACTH were relatively insoluble in citrate or phthalate buffers at the same pH (4.0) and ionic strength (0.1). Dialysis against citrate buffer for 2 days at 5° with an excess of solid protein gave a solution containing only 0.3 per cent protein; against phthalate buffer, less than 0.1 per cent protein could be dissolved. Moreover, the electrophoretic pattern in citrate (Fig. 1, *C*) was much like that observed in acetate or veronal on the alkaline side of the isoelectric point; *i.e.*, the main component was about 90 per cent of the total area. These experiments suggest a specific interaction of acetate and propionate ions and ACTH at certain pH values, which alters both the solubility and the electrophoretic pattern of the hormone. The unusual behavior of ACTH under these conditions was also found in the sedimentation experiments described below. However, in acetate at pH 2.7 (Fig. 1, *E*), the electrophoretic pattern was similar to that found on the alkaline side of the isoelectric point in that the major component represented about 90 per cent of the total area.

The mobility of the main component of our ACTH preparations is plotted at various pH values in Fig. 2. The isoelectric point was found to be at about pH 4.6, which is in good agreement with previous studies (2, 3). However, the mobility values are not entirely in accord with earlier measurements. The data given by Wilhelmi and Sayers (6) for swine ACTH and by Li *et al.* (2) for sheep ACTH have also been plotted in Fig. 2. It will be noted that our results differ from those given by Wilhelmi and Sayers who observed much more rapid migrations. While our determinations are in good agreement with those of Li *et al.* at values alkaline to the isoelectric point, they differ from those recorded by these investigators at more acid pH values.

Studies in Ultracentrifuge—These observations were made in the Spinco^a electrically driven ultracentrifuge, with the controls and procedures previously reported from this laboratory (7). All our studies were made at 59,780 r.p.m., which is equivalent to centrifugal fields of approximately $240,000 \times g$ and $300,000 \times g$ at the meniscus and base, respectively.

Measurements made in veronal buffer (0.1 M) showed a single sedimenting boundary (Fig. 3, A); similar results were obtained in solutions ad-

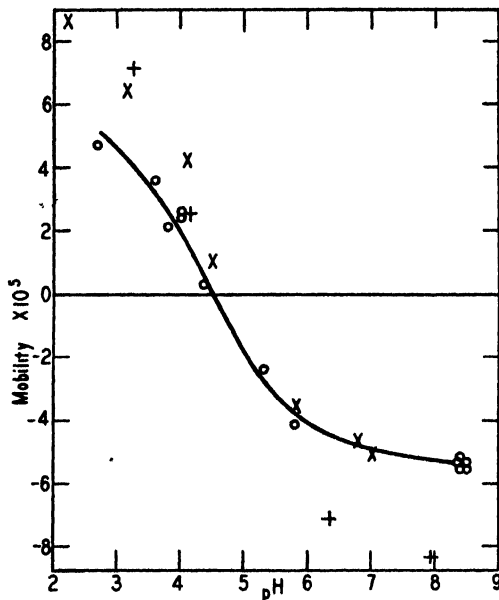


FIG. 2. Electrophoretic mobility as a function of pH of the main component of swine ACTH. The mobilities, in sq. cm. per volt per second, were calculated from descending patterns in univalent buffers at 1.5°. The pH values were estimated with a glass electrode at 25°. For comparison with our data, O, the results given by Wilhelmi and Sayers (6), +, and by Li, Evans, and Simpson (2), X, are also shown.

justed to pH 6.4 (Fig. 3, B) or to pH 9.3 in which the sodium chloride concentration was varied from 0.15 to 0.5 M. The sedimentation constants ($s_{20,w}$) for these runs are plotted against protein concentration in Fig. 4. It is apparent that the protein concentration does not influence the sedimentation rate. The average for the thirteen runs is $s_{20,w} = 1.92 \pm 0.045$ S (Svedberg units). It is of interest that the pH range includes conditions (pH 6.4) under which the hormone is stable, as well as more alkaline values (pH 8.5 to 9.3), at which the hormone is somewhat un-

^a Specialised Instruments Corporation, Belmont, California.

stable. Nevertheless, we have been unable to detect any differences in the sedimentation behavior of the hormone in these various media.

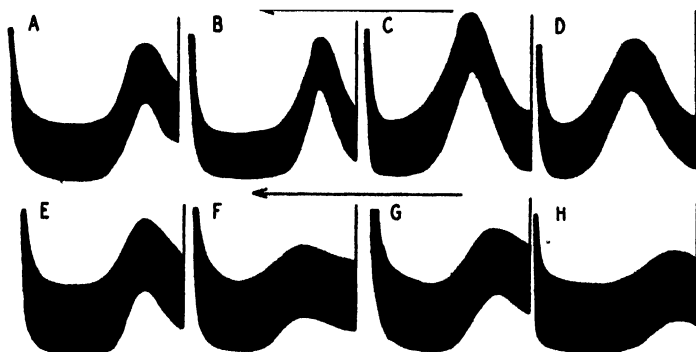


FIG. 3. Representative sedimentation patterns obtained on ACTH under different conditions. *A* is in 0.1 M veronal at pH 8.5 at a protein concentration of 0.87 per cent taken 102 minutes after attaining full speed. *B* is in 0.15 M NaCl at pH 6.4 (1.6 per cent) at 110 minutes. *C* is in 0.1 M acetate at pH 5.25 (1.95 per cent) at 132 minutes. *D* is in acetate at pH 4.1 (2.5 per cent) at 103 minutes. *E* is in 0.15 M NaCl + HCl at pH 0.2 at 97 minutes (Preparation 3, Table I). *F* is Preparation 5 of Table I at 135 minutes. *G* is Preparation 9 of Table I at 146 minutes. *H* is Preparation 12 of Table I at 126 minutes.

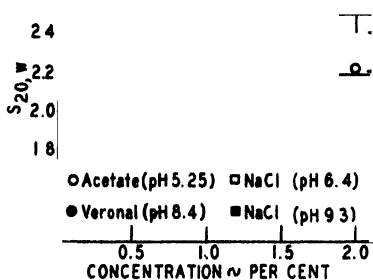


FIG. 4

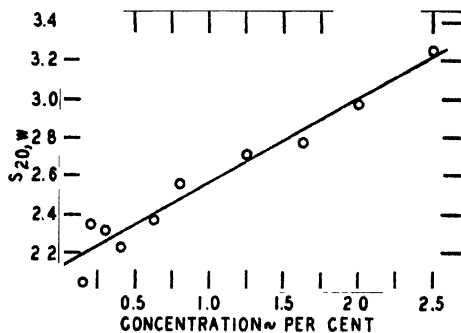


FIG. 5

FIG. 4. Sedimentation constant ($s_{20,w}$) in Svedberg units as a function of protein concentration.

FIG. 5. Sedimentation constant ($s_{20,w}$) in Svedberg units as a function of protein concentration as determined in 0.1 M acetate buffer at pH 4.1.

ACTH is stable and monodisperse (Fig. 3, *C*) at pH 5.25 in acetate buffer; yet the sedimentation rate found in this buffer is significantly different from the values obtained at more alkaline pH values (Fig. 4). Four determinations were made in which the protein concentration varied from

0.25 to 2.0 per cent. These runs, which were in excellent agreement, gave as an average value, $s_{20, w} = 2.19 \pm 0.015$ S.

Ultracentrifugal study of ACTH in acetate buffer at pH 4.1 gave results quite different from those already described. Whereas the hormone appears to be monodisperse over a wide range of protein concentration (an example is shown in Fig. 3, *D*), there is a marked effect of protein concentration on the sedimentation rate as indicated by the data for the eleven runs plotted in Fig. 5. It should be noted that the usual effect of increasing protein concentration is to diminish the sedimentation rate of an asymmetrical particle. Here the effect is the opposite, which suggests an aggregation of particles.

The straight line drawn in Fig. 5 was calculated by the method of least squares and is described by the equation, $s_{20, w} = 2.13 + 0.435C$, where C is the protein concentration. The extrapolated value at zero concentration is 2.13 S, which is in good agreement with the value of 2.19 S found in acetate buffer at pH 5.25. With the exception of the two points obtained at the lowest concentration of protein, at which the error in estimating sedimentation rate is quite high, the average deviation from the calculated line is about ± 2 per cent.

Observations were also made of the sedimentation behavior of ACTH after treatment at more acid pH values than those already given. These results are presented in Table I. It should be noted that no loss of biological potency of the hormone occurred even under the most acid conditions tested (1). Stability tests and sedimentation measurements were performed simultaneously on many of the same preparations used for the sedimentation measurements. Solutions as acid as pH 0.2 (Preparation 3) behaved as did those at less acid pH values when tested in the ultracentrifuge immediately following preparation (Fig. 3, *E*). After standing for 2 days at 5°, the substance (Preparation 5) was no longer monodisperse, since a considerable amount of more slowly sedimenting material could be detected (Fig. 3, *F*). Dilution of the same solution with the HCl + NaCl mixture (Preparation 6) gave a broad sedimenting boundary with $s_{20, w} = 1.61$ S; it is probable that this boundary represented a mixture of the original component and lighter material which could not be resolved.

ACTH which had been allowed to stand at about pH 0.1 gave sedimentation values of about half of those found at higher pH values. This was true whether the preparation was tested in a Lucite cell at the acid pH (Preparation 12, Fig. 3, *H*) or after neutralization with NaOH (Preparations 9 (Fig. 3, *G*) and Preparation 10). Thus, the breakdown of the protein in acid solutions does not appear to be reversible. The sedimenting area of these acid-treated preparations was found to be smaller than that expected for the amount of dissolved material; this suggests the occurrence

of a further breakdown into particles too small to be sedimented. It should be noted that the high ionic strength in Preparations 9 and 10 is due to the neutralization of the acid with NaOH; this introduced large corrections for the density and viscosity of the medium. However, the low

TABLE I
Sedimentation Behavior of Acid-Treated ACTH

The pH was measured with a glass electrode. The ionic strength was calculated from the NaCl content of the solution. The measurements were made at room temperature and $s_{20, w}$ was calculated for the average temperature during the time when photographs were taken. The usual corrections for density and viscosity of the medium have been made (8).

Preparation No.	Treatment and solvent	Ionic strength	pH	Concentration	$s_{20, w}$
				per cent	S
1	pH adjusted with HCl; allowed to stand overnight	0.15	1.0	1.5	2.21
2	Same as Preparation 1; pH raised with NaOH	0.15	3.5	1.35	2.13
3	pH adjusted with HCl	0.15	0.2	0.46	2.23
4	Same as Preparation 3; pH raised with NaOH	1.2	6.4	0.3	2.01
5	Same as Preparation 3; allowed to stand 2 days at 5°	0.15	0.2	0.46	2.11*
6	Same as Preparation 5; allowed to stand at 5° one more day, then diluted with solvent	0.15	0.15	0.23	1.61
7	pH adjusted with HCl; tested after standing overnight at 5°	0.15	2.8	1.0	2.26*
8	Same as Preparation 7; allowed to stand at 5° overnight and diluted with solvent	0.15	2.8	0.5	2.19†
9	At pH 0.1 overnight, pH then raised with M NaOH	0.77	7.8	0.3	1.11
10	At pH 0.1 overnight, pH then raised with M NaOH	0.88	6.7	0.6	1.03
11	At pH 1.0 overnight, then neutralized with M NaOH	0.12	6.5	0.75	1.99
12	Dissolved in 2 M HCl + 0.15 M NaCl	0.15	0.10	0.5	1.23

* Contained slowly sedimenting material in addition to main component.

† Main boundary was not homogeneous; there was also present some lighter material and a heavier component with $s_{20, w} = 4.0$.

sedimentation constants obtained in this manner were confirmed by measurements on Preparation 12 made at the strongly acid conditions.

From these observations and correlated stability studies of the biological activity of the hormone (1), there is no doubt that the protein can be de-

graded into molecules which are of much smaller size than the original hormone and which retain their activity. These results are in accord with claims that ACTH activity can be associated with substances of lower molecular weight than the proteins isolated by Li *et al.* (2) and Sayers *et al.* (3). It should be emphasized that the low average sedimentation constant ($s_{20, w} = 1.1$ S) observed by us at acid pH values represents determinations near the limit of resolution of the ultracentrifuge. Smaller units would probably escape detection unless present in high concentration, and heterogeneity would be difficult to estimate.

The average sedimentation constant for the preparations in Table I, which did not indicate substantial splitting of the particles, gave an average value of $s_{20, w} = 2.14 \pm 0.08$ S, which is in excellent agreement with the

TABLE II
Diffusion Measurements on ACTH

Solvent	pH	Concentration	$D_{20, w} \times 10^7$
		per cent	sq. cm. per sec.
Veronal.....	8.5	0.5	10.6
“.....	8.5	0.5	11.5
“.....	8.5	0.5	10.7
“.....	8.5	0.5	10.2
Acetate.....	5.0	0.6	10.3
“.....	5.0	0.6	10.7
“.....	4.7	0.4	10.9
“.....	4.7	0.4	11.1
“.....	4.8	0.3	11.5
“.....	4.8	0.3	10.2

observations at pH 5.25 ($s_{20, w} = 2.19$ S) and the extrapolated value at pH 4.1 ($s_{20, w} = 2.13$ S). The few earlier results obtained on ACTH are in accord with our measurements. Burtner (9) reported $s_{20, w} = 2.08$ S as the average of two determinations and the average of the three values given by Sayers *et al.* (3) is 2.06 S.

Diffusion Studies—Diffusion constants were determined in the 5.5 cm. electrophoresis cell from photographs taken by the schlieren scanning method as described by Longworth (10). The formula used was $D = (A^2)/(4\pi tH^2)$ where A is the area under the curve in sq. cm., H the maximum height of the boundary in cm., t the time in seconds, and D the diffusion constant in sq. cm. per second. Five runs were performed, each in duplicate, since the halves of the cell were used separately. Four to seven photographs were taken at intervals of about 10 to 50 hours. The runs were made at 1.5° in buffers at an ionic strength of 0.1 and the values for

$D_{20, w}$ were obtained by correcting in the usual way for temperature and viscosity differences (8). The results are given in Table II.

In veronal at pH 8.5 and in acetate at pH 4.7 to 5.0, values for $D_{20, w}$ were found to be identical. The average for all the measurements is $D_{20, w} = 10.8 \pm 0.4 \times 10^{-7}$ sq. cm. per second. This value is in good agreement with the single determination reported by Burtner (9) who found $D_{20, w} = 10.4 \times 10^{-7}$ sq. cm. per second.

DISCUSSION

Our electrophoretic data on ACTH appear to be similar to those previously reported. However, they have been interpreted somewhat differently than those presented in earlier studies. The patterns published by Li *et al.* (2) show asymmetry of the main boundaries and, in addition, a number of small boundaries. Li *et al.* have ascribed this to the fact "that the base-lines of these patterns are irregular owing to the use of a poor schlieren lens." If this interpretation is accepted, the claim for electrophoretic homogeneity is weak, since with such an instrument uncertainty would exist regarding the presence of migrating components which could amount to a substantial fraction of the total refractive area. The patterns published by Sayers *et al.* (3) also show asymmetry of the main component. Our interpretation that the asymmetry represents an additional component is supported by studies at pH values at which the different boundaries are definitely separated as shown in citrate buffer at pH 4.1 (Fig. 1, C). Nevertheless, the main component observed electrophoretically appears to be associated with ACTH activity. The biological activity of ACTH at various stages of purification was compared with the electrophoretic pattern, and it may be stated that the concentration of the minor components detected in our preparations at pH values on the alkaline side of the isoelectric point does not appear to possess any relationship to the potency of the preparation. It should also be noted that the similar biological and chemical properties described for sheep and swine ACTH are consistent with the view that the main component of these preparations is ACTH, particularly since such different methods of purification were employed for the isolation of the hormone from the glands of the two species (2, 3).

The marked electrophoretic heterogeneity of ACTH in acetate (or in propionate) near pH 4 poses an interesting problem. It is suggested that this may be due to a specific interaction of ACTH and acetate ions which causes a reversible aggregation of the primary particles. This is supported by our qualitative observations that the solubility of the protein is much greater in acetate or propionate buffers than in citrate or phthalate at the same pH and ionic strength. The marked effect of protein concentration on the sedimentation behavior of ACTH shown in Fig. 5 is also consistent

with this assumption. The aggregation and dissociation seem to be rapidly reversible, since observations made on solutions diluted from more concentrated ones and tested immediately were in agreement with those made on solutions dialyzed for several days and tested without dilution. In all these runs, no evidence was obtained for more than one sedimenting boundary, which would suggest that the boundary contains an equilibrium mixture of monomeric and aggregated forms of the protein.

Smith and Briggs (11) have recently reported that crystalline bovine serum albumin interacts with methyl orange to yield complex electrophoretic patterns in which the number of components depends on the concentration of the dye. They interpret this phenomenon as being due to reactive groups at various loci on the protein molecule, which possess quite different affinities for the dye. We may tentatively suppose that the ACTH-acetate interaction is similar, with the added factor that the presence of acetate causes an aggregation of the primary unit. The apparently greater homogeneity of ACTH in acetate at pH 2.7 compared to that at pH 4 may be assumed to be due to a more complete acetate-protein interaction. We hope to extend our studies by an electrophoretic and ultracentrifugal analysis of the effect of ionic strength, pH, and acetate concentration on these phenomena.

The molecular weight of ACTH may be estimated from our values for $s_{20, w}$ and D by the use of the usual formula (8) in which $M = RTs/D(1 - V\rho)$. Assuming that $V = 0.75$ and taking our average values, $D = 10.8 \times 10^{-7}$ and $s = 2.16 \times 10^{-13}$, we obtain $M = 19,400$ which is in good agreement with the value of 20,000 obtained by Burtner. We have preferred to use the value of $s_{20, w}$ obtained at acid pH values at which the hormone is stable rather than the value of $s_{20, w} = 1.92$ S which was found at neutral and alkaline pH at which the hormone is more labile. An adequate explanation for these differences in the sedimentation constant is not apparent at the present time.

Two important problems arise in connection with the nature of ACTH: first, the molecular size and biological activity of the hormone as it exists in the adenohypophysis, and, second, the characteristics of substances isolated by a variety of treatments from pituitary tissue. At the present time, there does not appear to be any unequivocal evidence against the idea that a protein of 20,000 molecular weight is the hormone normally stored in the gland. There have been a number of claims (4, 12-15) that ACTH activity is associated with smaller and more active units than the proteins isolated by earlier procedures of Li *et al.* (2) and Sayers *et al.* (3). Our sedimentation and stability studies in acid solutions provide support for these claims. However, it should be emphasized that these small active units are produced by the action of pepsin or by acid at low pH values.

The breakdown of the hormone under acid conditions appears to be a function of time, as indicated by some of our observations, and a function of temperature, as indicated by the observations of Li (16). It is rather striking that the small units liberated by treatment with acid appear to be more active than the parent protein (16). The report by Lesh *et al.* (15) that preparations of ACTH can be isolated following peptic digestion, which have 100 to 150 times the activity of the Armour standard, La-1-A, suggests that some caution is still necessary in associating ACTH activity with a particular chemical entity. It may be noted that the retention of biological activity by a protein which has been partially degraded is not a unique attribute of ACTH. One should recall the numerous studies on the hydrolysis of antitoxins by proteolytic enzymes, which have shown that units of half or smaller size retain all the potency of the original intact molecules (17, 18).

SUMMARY

Preparations of adrenocorticotrophic hormone (ACTH) of swine adeno-hypophysis have been studied electrophoretically and have been demonstrated to be relatively homogeneous on the alkaline side of the isoelectric point (pH 4.6); they contain 6 to 10 per cent of another component. In acetate buffer at about pH 4, the preparations show a number of components. This has been ascribed to a specific interaction of acetate and ACTH as suggested by the electrophoretic heterogeneity, the enhanced solubility, and a strong dependence of the sedimentation constant on the protein concentration. Under the other conditions studied, the sedimentation constant is independent of protein concentration. From the average values, $s_{20, w} = 2.16 \times 10^{-13}$ and $D_{20, w} = 10.8 \times 10^{-7}$ sq. cm. per second, the molecular weight is calculated to be 19,400.

In acid solutions, at about pH 2.8, the hormone may slowly dissociate and yield some material of lower molecular weight. At pH 0.1 to 0.2, this splitting is quite rapid and smaller sedimentation constants are observed; yet there is no loss of biological potency.

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TURNOVER RATES OF PHOSPHATE AND FATTY ACID MOIETIES OF PLASMA PHOSPHOLIPIDES*

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P^{32} -labeled plasma phospholipides have been used in this laboratory to measure turnover rates of circulating phospholipides. The labeled phospholipides were introduced directly into the circulation, and the rates of disappearance of their P^{32} from the blood stream were determined (1-3). It was recognized by us (4), as well as by others (5-7), that the rate at which the P^{32} is lost from this molecule may differ from the rate of loss of its oxidizable material. It is conceivable, for example, that after the phosphate group is split off other phosphate radicals, non-radioactive in this case, combine with the dephosphorylated residue at a more rapid rate than that at which the dephosphorylated residue itself is renewed. On the other hand, the α -glycerophosphate or α -glycerophosphorylcholine may serve as a relatively stable nucleus to which fatty acids become attached and from which they dissociate. Or the nitrogenous base may be renewed independently of the rest of the molecule. If any of the above reactions were to occur, the turnover rate of the phosphate radical would then differ from the turnover rates of either the fatty acid moiety or the nitrogenous base, or both.

It became of interest, therefore, to compare the turnover rates of the various parts of the phospholipide molecule. In the present study, we used plasma phospholipides in which the phosphate moiety was labeled with P^{32} and the fatty acid moiety with C^{14} . It is shown here that, when the turnover rates of intact plasma phospholipide are determined independently by each of these isotopes, similar values are obtained.

EXPERIMENTAL

Preparation of Doubly Labeled Plasma Phospholipides—The dog, designated here the donor dog, received, by stomach tube, 6 cc. of an aqueous solution of radioactive phosphate (675×10^6 c.p.m.). Immediately thereafter, the dog was fed, by the same method, 20 cc. of corn oil in which were dissolved 365 mg. of tripalmitin containing palmitic acid labeled with C^{14}

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at the carboxyl carbon. The administered tripalmitin¹ had an activity of 42×10^6 c.p.m. 10 hours later, a sample of blood was removed for analysis of plasma phospholipides and non-phospholipides. After 24 hours the dog was exsanguinated, its heparinized blood centrifuged, and the plasma separated.

Turnover Experiment—64 cc. of the plasma containing the doubly labeled phospholipides were injected intravenously into each of two dogs (designated here recipient Dogs A and B). This was carried out within 30 minutes after the separation of the plasma. Samples of blood were removed from both dogs at several time intervals, and the plasma was analyzed for the specific activities of its phospholipide phosphorus (PL-P) and its phospholipide fatty acid carbons (PLFA-C).

Methods of Analyses—The extraction of lipides from plasma and their separation into a phospholipide (Fraction A) and a non-phospholipide fraction (Fraction B) were carried out as described by Goldman *et al.* (10).

In the case of the recipient dogs, only Fraction A was considered further, while both fractions were further analyzed in the donor dog.

Fractions A and B were saponified (10), the saponified material was acidified with 5 N H_2SO_4 , and the mixture was cooled in a refrigerator. The fatty acids were collected on fritted glass filters and washed several times with small amounts of a 5 per cent Na_2SO_4 solution. They were then dissolved in acetone, the acetone was evaporated, and the fatty acids were taken up again by ethyl ether. Aliquots were oxidized to CO_2 , and BaCO_3 mounts were prepared by the technique of Entenman *et al.* (11).

To analyze Fraction A for its phosphorus content, the filtrates and washings (after collection of the fatty acids on the fritted glass filters) were combined. An aliquot was mounted on a blotter for determination of P^{32} , and total phosphorus was determined on a different aliquot by King's method (12).

Results

Table I shows the degree of labeling of the phospholipide and non-phospholipide fatty acids of the donor dog at 10 and 24 hours after the administration of the isotopic compounds. It can be seen that, at the later interval, the specific activity of the phospholipide fatty acids was more than 10 times that of the non-phospholipide fatty acids.

¹ The labeled palmitic acid was synthesized as described by Dauben (8), and the triglyceride was prepared by a modification of the method of Feuge *et al.* (9).

Fig. 1 shows the specific activities of plasma PL-P and PLFA-C³ at various time intervals after the injection of the doubly labeled phospholipides into the recipient dogs.

TABLE I
Specific Activities of Non-Phospholipide Fatty Acids and Phospholipide Fatty Acids of Donor Dog

Hrs. after administration	Specific activity of	
	Non-phospholipide fatty acid carbon	Phospholipide fatty acid carbon
	c.p.m. per mg C	c.p.m. per mg. C
10	160	396
24	41	520

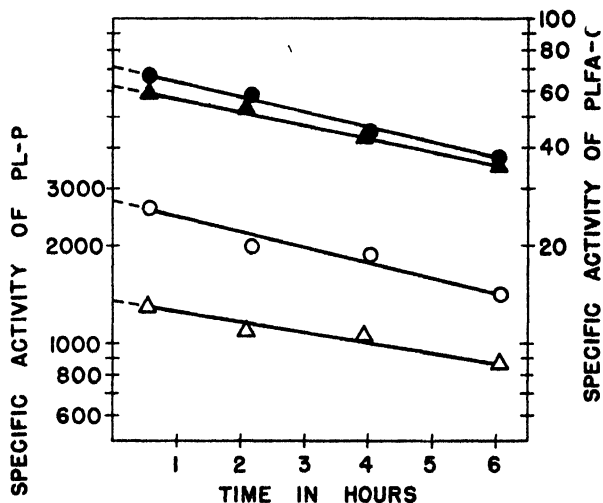


FIG. 1. The disappearance of labeled phospholipides from the circulation. The specific activities are expressed as counts per minute per mg. of PL-P and counts per minute per mg. of PLFA-C. Dog A, PL-P (○); Dog B, PL-P (△); Dog A, PLFA-C (●); Dog B, PLFA-C (▲).

An inspection of the curves indicates that the PL-P curves and the PLFA-C curves are close to parallel in each of the two dogs. Such parallelism would indicate that the ratio of the PL-P specific activity to the

* Although the administered plamitic acid contained C¹⁴ only in its carboxyl carbon, the isolated fatty acids are a mixture of the various fatty acids found in the blood, and the combustion of these acids yields a specific activity representing the average for all fatty acid carbons.

PLFA-C specific activity remained constant and that, therefore, both labeled fractions of the phospholipide molecule disappeared from the blood stream simultaneously. This is shown more precisely in the following calculations for turnover times and turnover rates of the labeled moieties.

As pointed out previously (2, 3), the turnover time (t_i) may be calculated from the expression $t_i = 1.44t_{1/2}$, where $t_{1/2}$ is the half time; i.e., the time at which the specific activity of a substance is exactly half of its specific activity at zero time.

The turnover rate, p , may then be expressed as $p = r/t_i$, where r represents the total amount of the substance in the entire circulating fluid. The value for r may be obtained by dividing the total counts injected by the specific activity at zero time.

TABLE II
Turnover Times and Turnover Rates of Phospholipide Phosphorus and Phospholipide Fatty Acid Carbon of Dog Plasma

Dog	Weight	Turnover time of		Turnover rate of		Turnover rate of whole phospholipide molecule	
		Phospholipide phosphorus	Phospholipide fatty acid carbons	Phospholipide phosphorus	Phospholipide fatty acid carbons	Based on phospholipide phosphorus	Based on phospholipide fatty acid carbons
	kg.	hr.	hr.	mm P per hr.	mm C per hr.	mm phospholipide per hr.	mm phospholipide per hr.
A	8.6	9	9	0.13	4.8	0.13	0.14
B	12.9	13	11	0.18	4.5	0.18	0.14

Turnover Times—Values for turnover time of PL-P and PLFA-C are recorded in Table II. The values for phosphorus are in good agreement with those reported in earlier investigations (1-3, 13).

Turnover Rates—The turnover rates for the phosphorus and fatty acid carbons of plasma phospholipides are also shown in Table II. If the turnover times are alike, the turnover rate per millimole of carbon must, of course, be faster than that of the phosphorus, since each phospholipide molecule contains only 1 atom of phosphorus but a much larger number of fatty acid carbons.

To compare these two experimentally determined values we have, therefore, calculated from each the turnover rate of the *whole phospholipide* molecule.

The turnover rate of the PL-P (millimoles of P turned over per hour) is, of course, the same as the rate of the entire molecule (Table II).

In order to compute the turnover rate of the whole phospholipide from

that of its PLFA-C, it is, however, first necessary to know the average number of fatty acid carbons per phospholipide molecule. This value may be calculated in the following manner.

The factor 25 was used to convert mg. of phosphorus to mg. of phospholipide. On the basis of this factor the average molecular weight of a phospholipide is 25×31 or 775. If lecithin were the only phospholipide present, the average molecular weight of a fatty acid would, therefore, be 277. If only sphingomyelin were present, this value would be 329. Since, however, dog plasma contains about 5 molecules of lecithin for 1 molecule of sphingomyelin,² it can be calculated that the average molecular weight of a phospholipide fatty acid is 286, which is equivalent to 18.2 carbon atoms per fatty acid molecule.

On the basis of the above distribution of lecithin and sphingomyelin in plasma phospholipides, it follows that 6 phospholipide molecules contain 11 fatty acid molecules, or that there are 1.83 fatty acid molecules per phospholipide molecule. The average number of fatty acid carbons per phospholipide molecule is, therefore, obtained by multiplying 1.83×18.2 and is 33.3. Then the (turnover rate of PLFA-C)/33.3 yields the turnover rate of the phospholipide itself.

The values for turnover of whole phospholipide molecules, based on the above calculations, are recorded in the last column of Table II.

A comparison of the results shows that similar values are obtained, regardless of whether the calculations were based on P³² or on C¹⁴ (Table II).

It should be noted that the values given here represent averages for the various types of phospholipides contained in plasma. Since Zilversmit *et al.* (13) have demonstrated that plasma lecithins are turned over at a rate which is about 5 times as great as that of plasma sphingomyelin, and since dog plasma contains 5 times as much lecithin as sphingomyelin, the measurements made in this study represent, primarily, lecithin. The only labeled fatty acid administered to the donor dog was palmitic acid. It is, however, quite reasonable to assume that, 24 hours after the injection, other fatty acids were also labeled with C¹⁴. Even though fatty acids other than palmitic, in the injected phospholipides, were probably labeled, palmitic acid undoubtedly had the highest specific activity. It has already been pointed out that the isolated fatty acids consisted of a mixture of all the phospholipide fatty acids found in plasma, and that combustion

² Dog plasma contains little cephalin (14-17). Similar observations have also been reported for human plasma (14, 16-18), but Albrink (19) has reported choline to phosphorus ratios of 0.7 to 0.8 in human serum phospholipides. Her finding indicates the presence of phospholipides which do not contain choline and are presumably cephalins.

of these fatty acids to CO_2 yielded a specific activity which represents an average value for all fatty acid carbons.

Studies in this laboratory have shown that plasma phospholipides are synthesized to a large extent in the liver and that this organ is also the chief site of their destruction (10, 20). The fact that the phosphate moiety is turned over at the same rate as the fatty acid moiety rules out the possibility that our earlier measurements with P^{32} dealt merely with the exchange of phosphate radicals, or that a phosphorus-containing compound serves as a stable nucleus on which fatty acids are being constantly renewed. Even though data on the turnover of the nitrogen moiety, comparable to those recorded here for the phosphate and fatty acid moieties, are not available, the results presented here do indicate that the entire phospholipide molecule is removed as a unit from the blood stream, and that exchange reactions do not occur either in the blood or as the phospholipide molecules pass through the liver.

SUMMARY

1. Plasma, containing phospholipides in which the phosphate and fatty acid moieties were labeled with P^{32} and C^{14} , respectively, was injected intravenously into two dogs, and the rates of disappearance of these isotopes from the circulating phospholipides were used to measure their turnover rates.

2. Plasma phospholipides were turned over at a rate of 0.13 and 0.17 mm per hour in Dogs A and B, respectively, as judged by P^{32} measurements. The rate was 0.14 mm per hour when based on C^{14} -fatty acid determinations.

3. The significance of the similarity in the turnover rates of these two moieties of plasma phospholipides is discussed.

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NIACIN AND THE ABILITY OF CYSTINE TO AUGMENT DEPOSITION OF LIVER FAT

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As a preliminary to further work on the problem of dietary fatty livers, it seemed desirable to repeat the work of Beeston and Channon (1) on the augmentative influence of cystine on the lipide content of livers of rats receiving a diet low in protein (casein) and high in fat. Much to our surprise, preliminary experiments failed to support Beeston and Channon, although previous data from this laboratory by Tucker and Eckstein (2) were in full accordance with the report of the English investigators. During the time intervening between the study made by Tucker and Eckstein and the present one, the source of the vitamin B complex used routinely in our laboratory was changed from dried brewers' yeast to a mixture of pure B vitamins. This mixture was employed in the preliminary experiments. For this reason, other rats were placed on a diet containing yeast as the source of the B vitamins; under these conditions the observations of Beeston and Channon were reconfirmed. On the basis of the daily intake of the rat, a comparison of the analysis of the dried yeast and the composition of our mixture of B vitamins revealed that the niacin content of the latter was significantly higher. A new mixture of pure B vitamins, in which the niacin content was reduced to approximately that of yeast, was prepared. Rats receiving this mixture of vitamins in the diet showed the expected cystine effect. In an additional experiment, a comparatively large amount of tryptophan was added to the diet and the niacin omitted. Under these conditions, we again failed to find the augmentative effect of cystine on liver lipide content.

EXPERIMENTAL

Young male rats of the Wistar strain in litter units, weighing 120 to 160 gm., were kept for 3 weeks on low protein, high fat,* choline-free diets (Table I). Half of the animals of each litter received a diet supplemented with 0.5 per cent L-cystine. Records of food intake were kept and the animals were weighed weekly. At the end of the feeding period, the rats were killed by decapitation and their livers excised, weighed, and subsequently analyzed for total lipides by a slight modification of the method of Tucker and Eckstein (2). Comparisons were made between the liver

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lipide content of animals of the "control" and "cystine" series (Series A and B, respectively), with the Student-Fisher *t* test as the criterion of significance (3). Table II summarizes the four types of experiments conducted. The several groups shown in Table II represent duplications of the experiments carried out at different times.

In Experiment I, Table I (in which Diets I-A and I-B were used), the vitamin B complex was supplied as the mixture of pure B vitamins (desig-

TABLE I
Composition of Diets

The diets listed below are all of the "control" or A series; corresponding diets of the "cystine" or B series contained 0.5 per cent L-cystine, added at the expense of glucose.

Component	Diet I-A	Diet II-A	Diet III-A	Diet IV-A
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein, Labco, "vitamin-free"	5	5	5	5
Lard	40	40	40	40
Glucose monohydrate, Cerelose	46	47	46	45.8
Salt mixture*	5	5	5	5
Ruffex, Fisher	3	3	3	3
Supplement,† vitamin B Mixture I	1	0	0	0
" " " " II	0	0	1	0
" " " " III	0	0	0	1
" yeast	0	‡	0	0
DL-Tryptophan	0	0	0	0.19

* Osborne and Mendel (4).

† The mixtures of pure vitamins differed only in their content of niacin. In 100 gm. of each mixture were contained 37 mg. of thiamine hydrochloride, 37 mg. of pyridoxine hydrochloride, 75 mg. of riboflavin, 300 mg. of calcium *d*-pantothenate, and 300 mg. of *p*-aminobenzoic acid. In addition, Mixture I contained 375 mg. of niacin, Mixture II contained 150 mg. of niacin, and Mixture III lacked niacin entirely. In each case, the balance of the mixture was corn-starch. Vitamins A and D were supplied by a daily drop of cod liver oil placed on top of the diet of each rat.

‡ Rats on Diet II received the vitamin B complex in a daily yeast tablet placed on top of the diet of each rat.

nated as vitamin B Mixture I) usually used in this laboratory. The other constituents of the diet were the same as in the experiments of Tucker and Eckstein, except that Ruffex was substituted for agar as bulk. Under the conditions of Experiment I the cystine effect did not occur. The average per cent lipide in the livers of the control rats was 30.8, of the cystine-fed rats, 33.0. This difference is not statistically significant.

Experiment II clearly demonstrated that cystine augments liver lipide content when dried yeast is substituted for vitamin B Mixture I in the diet. The livers of the control animals in this experiment contained an

TABLE II

Liver Lipide Content of Rats on Diets Described in Table I

The values in parentheses are ranges for the individual animals.

Group No.	Control diet	No. of rats	Fresh liver		Group No.	Cystine diet	No. of rats	Fresh liver	
			Weight	Total lipides				Weight	Total lipides
Experiment I (high niacin supplement)									
1-A	I-A	5	8.1	29.4 (17.0-38.8)	1-B	I-B	6	10.2	35.6 (27.0-39.8)
2-A	I-A	5	9.4	32.1 (27.9-39.5)	2-B	I-B	6	9.7	30.9 (12.8-39.3)
3-A	I-A	9	9.7	30.9 (18.8-41.9)	3-B	I-B	7	10.4	32.5 (21.3-46.3)
Average				30.8*					33.0*
Experiment II (yeast supplement)									
4-A	II-A	9	8.7	32.0 (20.4-43.2)	4-B	II-B	9	11.7	36.3 (24.0-42.5)
5-A	II-A	8	7.5	28.3 (14.7-42.5)	5-B	II-B	7	9.9	38.0 (27.6-43.9)
Average				30.2†					37.1†
Experiment III (low niacin supplement)									
6-A	III-A	9	7.1	31.2 (16.3-41.7)	6-B	III-B	8	11.4	37.2 (26.0-46.3)
7-A	III-A	9	8.3	33.7 (21.9-44.4)	7-B	III-B	9	11.5	41.1 (38.6-44.3)
8-A	III-A	7	8.3	36.2 (33.6-40.4)	8-B	III-B	7	9.9	39.7 (34.1-48.5)
Average				33.5‡					39.4‡
Experiment IV (tryptophan supplement, no niacin)									
9-A	IV-A	9	7.8	34.5§ (25.9-42.1)	9-B	IV-B	9	8.9	35.9§ (22.9-42.6)

* Student-Fisher t for these data = 0.820; $P \sim 0.5$; thus the difference between the averages is insignificant.

† For these data, $t = 2.83$; $0.01 < P < 0.001$; the difference between the averages is significant.

‡ For these data, $t = 3.36$; $P \sim 0.01$; the difference between the averages is significant.

§ For these data, $t = 0.503$; $P \sim 0.5$; the difference between the averages is insignificant.

average of 30.2 per cent lipide, whereas the livers of the cystine-fed rats contained 37.1 per cent lipide. This difference is statistically significant and in agreement with the original report by Beeston and Channon.

The effect of niacin in dietary fatty livers was considered worthy of further investigation. In Experiment III, a mixture of pure vitamins was again employed, but, in this case, the niacin content was reduced to a level corresponding to that furnished by the yeast in Experiment II. Under these conditions, the increase in liver lipide content due to cystine was of the same order of magnitude as in Experiment II: control rats 33.5 per cent liver lipide, cystine-fed rats, 39.4 per cent, a statistically significant difference.

Experiment IV was designed to determine whether the addition of tryptophan to a niacin-free diet would result in the same effect as the incorporation of the generous amount of niacin employed in Diets I-A and I-B. Diets IV-A and IV-B contained 0.25 per cent of tryptophan, a level which is well above the amount (0.2 per cent) required for growth of the white rat (5). According to Krehl *et al.* (6), tryptophan can be substituted for niacin in the diet of the growing rat in the proportion of 50:1. In Experiment IV, the diet contained no niacin but a generous amount of tryptophan, comparable on the basis of this relationship to the high level of niacin in Diet I. The livers of the control animals contained 34.5 per cent lipide, and the cystine-fed animals, 35.9 per cent. This difference is not statistically significant; hence the cystine effect does not occur under these conditions.

A study of the data on food intake and change in body weight indicated that neither one of these factors is responsible for the results reported herein.

SUMMARY

1. The niacin (or tryptophan) content of the diet has been shown to influence the ability of cystine to augment the liver lipide content of rats fed a low protein, high fat, choline-free diet.

2. In the presence of "high" (generously adequate) amounts of niacin or tryptophan, cystine had no effect on the accumulation of lipide in the liver.

3. Cystine exerted a positive effect on the accumulation of fat in the liver only when the dietary content of niacin was relatively "low."

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THE SURFACE CHEMISTRY OF BONE*

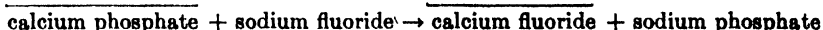
II. FLUORIDE DEPOSITION

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From solubility considerations, the fluoride taken up by teeth from topically applied solutions (usually 1 to 2 per cent NaF) is fixed by a mechanism involving a double salt decomposition



Ample experimental evidence for this mechanism has appeared in the literature (1-4). On the other hand, the fluoride ordinarily found in the calcified structures, derived from the ingestion of fluoride-containing water and food, has been taken up from solutions of low concentrations (a few parts per million or less). Since, in this case, the solubility of calcium fluoride is not exceeded, some other mechanism must be operative. Gerould (4) has stressed this point and showed that calcium fluoride as such is not found even in fluorosed teeth. He offered evidence that the fluoride was present in the apatite structure.

The use of bone and synthetic apatites as agents for the reversible adsorption of fluoride from drinking water on a commercial scale (5-7) suggests that this second mechanism may involve ionic exchange.

Recently, a series of investigations (8-10) has demonstrated that the skeletal fixation of uranium involved an exchange of calcium for uranium in the crystal surfaces of the mineral phase of bone. Uranyl ions were in direct competition with calcium ions for the surface phosphate groups. By means of the techniques employed in the uranium studies *in vitro*, i.e., ion exchange and ion competition, the mechanism of fluoride fixation by bone mineral has been investigated. The evidence obtained indicates that, at low concentrations, fluoride ion does not displace phosphate but rather hydroxyl and bicarbonate groups in the mineral surfaces.

* This paper is based on work performed under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, New York. It was originally presented before the meeting of the American Chemical Society, Detroit, 1950

EXPERIMENTAL

Methods—Fluoride was determined by a modification (11) of the method of Willard and Winter (12). Phosphate was analyzed according to Fiske and Subbarow (13) and calcium by the method of Roe and Kahn (14). Radioactivity measurements of P^{32} and Ca^{45} were made with a thin, mica window Geiger tube equipped with a standard scaling circuit (15). Sufficient counts were accumulated to keep the error of radioactivity measurement 3 per cent or less.

Bone, taken from the shafts of the long bones of adult rabbits, was freed of soft tissue and ashed by boiling in alkaline glycol (16). After being washed and dried, the ash was ground to a particle size between 60 and 120 mesh.

Effect of Fluoride Impregnation on Calcium and Phosphate Exchange—When powdered bone is equilibrated with solutions containing radioactive phosphate or calcium, it is possible, by means of standard isotope dilution techniques, to determine the amount of these ions contained in the mineral surface (9). If fluoride ions in combining with bone mineral displaced phosphate groups, it could be expected that the amount of surface or exchangeable phosphate would be markedly reduced by fluoride impregnation.

Glycol-ashed bone was impregnated with fluoride by shaking the powdered material with 0.0004 M NaF solution for 4 days at room temperature, pH 7.0 to 7.3. To attain maximum impregnation and yet avoid exceeding the solubility of CaF_2 , it was necessary to keep the fluoride concentration low and overcome this disadvantage by equilibrating small amounts of bone with large volumes of fluoride solution (0.25 mg. of ash per ml. of solution). These conditions were found to be optimal by preliminary experiment. Control specimens were given identical treatment, except NaCl was substituted for NaF. Following the impregnation, the solutions were aspirated and the ash analyzed for fluoride content. It was found to contain 8.4 mg. of F per gm.

20 mg. of this impregnated bone ash were equilibrated with 10 ml. of 0.001 M phosphate buffer containing P^{32} at pH 7.3 for 4 days at room temperature. The results of this study are presented in Table I.

It is evident that fluoride impregnation did not significantly alter the amount of surface phosphate. Had fluoride displaced phosphate on a mole per mole basis, the exchangeable phosphate would have been reduced from 12.9 to 3.9 per cent.

Similar experiments were performed to determine the effect of fluoride impregnation on calcium exchange. In this case, to avoid exceeding the solubility of CaF_2 , relatively large volumes of dilute (0.0005 M) $CaCl_2$ solution containing Ca^{45} were equilibrated with small amounts of ash,

i.e. 0.25 mg. per ml. Although unbuffered, the solutions maintained a pH of 6.7 ± 0.2 . The exchange was studied at intervals up to 6 days, at which time a quasi-equilibrium had been reached. The results, given in Fig. 1, show that the amount and rate of radiocalcium uptake were unaltered by fluoride impregnation. The equilibrium exchange values are given in Table I.

TABLE I
Effect of Fluoride Impregnation on Phosphate and Calcium Exchange

Ion tested	Per cent exchange*	
	Control	F-impregnated
Phosphate	12.9 ± 0.3	12.2 ± 1.5
Calcium	13.8 ± 1.4	14.3 ± 0.4

* Four samples equilibrated 4 days were averaged. Both the mean and the average deviation of the mean are given.

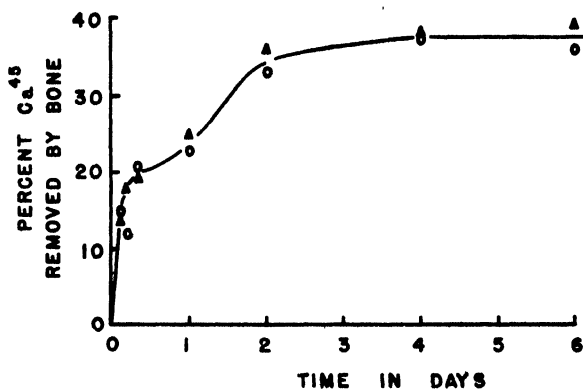


FIG. 1. The lack of effect of F impregnation on calcium exchange. O, control values; ▲, values obtained with fluoride-impregnated bone.

For purposes of comparison, the calcium exchange of CaF_2 was also studied. Both reagent grade CaF_2 (Baker's Analyzed) and a preparation made by mixing equivalent amounts of CaOH and HF were tested. Under the conditions described above for the equilibration of impregnated ash, no exchange of calcium was detectable with either preparation.

Thus, the exchange results suggest that fluoride impregnation does not involve the displacement of phosphate or the precipitation of CaF_2 .

Ion Competition Experiments—If phosphate, as suggested above, is not replaced during the fixation of fluoride by bone, very few possibilities remain. Of the non-phosphate anions found in bone mineral, only hy-

droxyl and carbonate (or bicarbonate) are present in appreciable quantities. Unfortunately, it is exceedingly difficult to perform exchange studies with these ions in a fashion similar to that described above for phosphate. It was necessary, therefore, to apply a different technique.

A series of ion competition experiments was conducted in which the effect of various ions on fluoride uptake was determined. Bone ash was added in the proportion of 1 mg. per 10 ml. to solutions containing some or all of the following: NaF, NaHCO₃, CaCl₂, and sodium phosphate buffer in various concentrations. After equilibration with 95 per cent O₂-5 per cent CO₂ at atmospheric pressure, the suspensions were sealed and shaken at room temperature for 4 days, at which time an apparent equilib-

TABLE II
Composition of Solutions Used in Ion Competition Studies

Variable studied	Fluoride added initially	Equilibrium concentrations			
		P	Ca	OH	HCO ₃
	<i>γ per ml.</i>	<i>μM per ml.</i>	<i>μM per ml.</i>	<i>μM per ml.</i>	<i>μM per ml.</i>
P:Ca	5	8.4	178	0.3	25,000
	5	42.0	29	0.3	25,000
	5	1030	5.8	0.3	25,000
HCO ₃	5	42	29	0.3	25,000
	5	52	45	0.3	50,000
	5	68	28	0.3	125,000
OH	10	970	20	0.01	*
	10	970	3.2	0.2	*
	10	970	1.0	8.9	*
	10	970	1.0	50.0	*

* Not equilibrated with 95 per cent O₂-5 per cent CO₂.

rium had been established. The supernatant solutions were aspirated and the bone analyzed for fluoride content.

The concentrations of the various ions at equilibrium are given in Table II. Bicarbonate and hydroxyl ions were varied independently. It is not possible to vary calcium and phosphate independently in the presence of bone. At equilibrium, high calcium values are associated with low phosphate concentrations and vice versa. Therefore, in Table II variations in the P:Ca ratio are recorded.

The effect of these changes in the composition of the solution on fluoride uptake by bone is presented in Fig. 2. From these data, it is clear that variation in either the bicarbonate or hydroxyl concentration markedly affected the uptake of fluoride by the bone ash. On the other hand, wide fluctuations of the P:Ca ratio in the equilibrating fluid were without significant effect.

Exchange Capacity of Ashed Bone—It was of interest to determine the maximum amount of fluoride which ashed bone can remove from solution by exchange. Approximately 300 mg. of ash were placed in a small sintered glass filter stick. Fluoride solution (2 γ of F per ml. as NaF) was allowed to enter from the bottom and, after passing through the column of bone ash, to escape through a side arm leading to a collection flask. The rate of flow was somewhat variable, averaging 5 ml. per hour. A total of 9.5 liters was passed through the column. Analyses for fluoride were performed at intervals on the effluent solution and on the bone ash at the conclusion of the experiment.

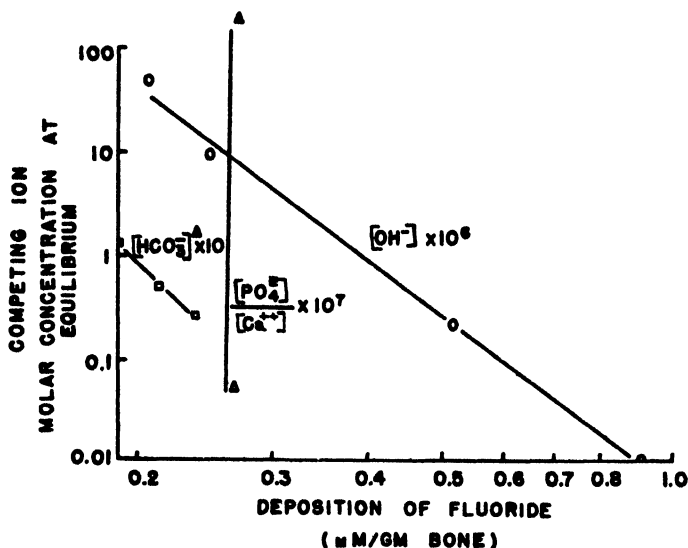


Fig. 2. Results of ion competition

In the beginning, the effluent solution contained only 20 per cent of the fluoride originally present (80 per cent removed by the bone). Gradually, the concentration of fluoride rose until, after 9.5 liters had passed through the column, the concentrations of fluoride in the affluent and effluent were equal.

The bone ash was sucked dry and analyzed. It contained 1.04 per cent fluoride.

DISCUSSION

In the experiments described above fluoride was transferred from solution to bone, although in every instance the product $[Ca^{++}] \times [F^-]^2$ was less than 5×10^{-12} . Both the rate and extent of radiocalcium exchange were unaffected by the fluoride impregnation. Clearly, the fixation proc-

ess did not involve a precipitation of calcium fluoride. The studies with radiophosphate showed that fluoride did not replace surface phosphate by exchange adsorption. Ion competition studies offered confirmatory evidence, since fluoride uptake by bone was not influenced by large variations of the Ca:P ratio in the solution phase. There was, however, a direct competition between hydroxyl, bicarbonate, and fluoride ions for combining sites in bone. A lack of information on the activity coefficients of these ions when bound in the solid phase precludes any mathematical analysis of the results. Nonetheless, the case for exchange adsorption (heteroionic exchange) as the mechanism of fluoride deposition seems well established.

When dilute fluoride solution is passed through a column of powdered, ashed bone, the reaction follows a course typical of an exchange adsorption, showing a maximum fluoride uptake of 1.04 per cent. If it is assumed that the ash is represented by the general formula $\text{Ca}_6(\text{PO}_4)_3 \cdot \text{CaX}_3$, where X may be OH^- , F^- , or HCO_3^- , this constitutes a maximum substitution by fluoride of one-fourth of the total number of X positions. The availability (surface location) of the X positions corresponds fairly well with previous studies on the calcium and phosphate fractions found in the mineral surfaces, approximately one-fifth (17) and one-sixth (18) respectively.

Thus, these results offer a sound experimental basis for the use of bone ash and synthetic apatites as reversible exchange adsorbers for fluoride on a commercial scale (5-7).

Though experimental evidence is almost totally lacking, it has been generally assumed that the CO_2 found in bone is present in the form of carbonate. In these experiments, the CO_2 in solution (as bicarbonate) was in competition with two *monovalent* ions, fluoride and hydroxyl. Since it was shown that no CaF_2 was formed as such, the competition must be formulated as $\text{BOCO}_2\text{H} + \text{F}^- \rightarrow \text{BF} + \text{HCO}_3^-$ (where B represents cationic bone), indicating that CO_2 is bound to the mineral by a single bond. It is possible, however, that the CO_2 is actually present as carbonate because of further ionization, i.e. $\text{BOCO}_2\text{H} + \text{M}^+ \rightarrow \text{BOCO}_2\text{M} + \text{H}^+$ (where M^+ represents a cation, probably sodium ion). Further studies on this interesting problem are desirable.

SUMMARY

The mechanism of fluoride deposition in bone was investigated by means of isotopic exchange, ion competition, and column techniques. It was demonstrated that fluoride replaces either hydroxyl or bicarbonate ions in the surfaces of the mineral phase.

The technical assistance of B. J. Mulryan is gratefully acknowledged.

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OXIDATION OF ASPARTIC ACID BY RAT LIVER*

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During the course of a study of the effect of amino acids on fatty acid catabolism by liver it was noted that in washed particles of rat liver, fortified with adenosinetriphosphate (ATP), L-aspartic acid caused an increase in the oxygen consumption of such magnitude as to indicate a substantial oxidation of the amino acid. Since this behavior seemed at variance with recent findings of Ratner and Pappas (1) and with observations of Still *et al.* (2) with the "cyclophorase" system of the rabbit, a closer investigation of aspartic acid oxidation was undertaken. This study has shown that L-aspartic acid (but not the D isomer) is readily oxidized by washed homogenates of rat liver, that adenine nucleotide is necessary, and that the reaction is inhibited completely or nearly so by malonate, arsenite, and *trans*-aconitate and by freezing of the tissue or treatment with distilled water. The results of this study are interpreted to indicate that the oxidation of L-aspartate in rat liver is not due to a specific oxidase, but is the result of transamination of aspartic acid to yield oxalacetate which is subsequently oxidized by way of the Krebs cycle.

Results

The addition of aspartic acid to a washed homogenate of rat liver fortified with ATP results in a rapid uptake of oxygen, which is linear for about 25 to 30 minutes and drops off thereafter to reach the endogenous level in about 40 minutes. A typical result is given in Fig. 1. The oxygen uptake was variable in different livers, but in the majority the net uptake in 1 hour for 500 mg. of fresh liver ranged between 6 and 10 μ M. This rate of oxidation is rather low, being about one-half to two-thirds that displayed by octanoate or members of the citric acid cycle. Adenylic acid will also activate the oxidation of aspartic acid, but not quite to the extent of ATP. The drop in oxygen uptake occurring after 25 minutes is not due to depletion of the substrate, nor is it due to ATP depletion, since addition of this substance after the initial rapid oxygen uptake ceased did not reactivate the system.

* Aided by grants from the National Cancer Institute, the United States Public Health Service, and the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

Except for the formation of glutamic acid, to be discussed later, oxidation appeared to be complete; there was no accumulation of ketone bodies, α -keto acids, citric acid, or lactic acid.

Specificity—Activity in the washed, fortified homogenate is restricted entirely to the natural L(+) isomer (Table I). At a high concentration of

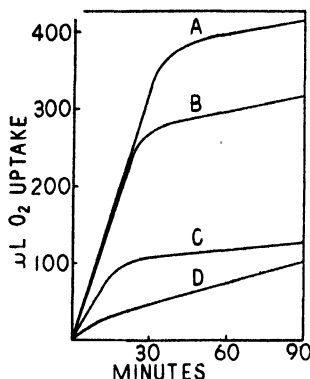


FIG. 1. Oxidation of aspartic acid by washed rat liver homogenate. Curve A, complete system with ATP; Curve B, complete system with adenylic acid; Curve C, ATP or adenylic acid, but no substrate; Curve D, no nucleotide, with and without substrate.

TABLE I
Specificity of Substrate

The oxygen uptake values are corrected for endogenous respiration and are given in microliters. The D, DL, and L forms of aspartic acid were used in Experiments 1, 2, and 3.

Concentration	Experiment 1			Experiment 2			Experiment 3	
	D	DL	L	D	DL	L	DL	L
M								
0.001						92		126
0.002					82		128	
0.005	6	98	101	8	129	129	196	196

aspartate, both the DL and the L forms exhibited identical rates of oxygen uptake, whereas the D form was entirely inactive. At lower concentrations the rate of oxidation of DL-aspartate was approximately equal to that of L-aspartate at one-half the concentration. Apparently the D-aspartate oxidase, found by Still *et al.* (2) in the cyclophorase system of the rabbit, is either absent from rat liver or has been removed or inactivated during the preparation of the tissue.

Effect of Substrate and Tissue Concentration—Within rather wide limits oxygen uptake was found to be proportional to the concentration of tissue (Fig. 2). The absence of a dilution effect, such as would be expected if a soluble enzyme or cofactor was diffusing, suggests that the enzymes concerned with the oxidation of aspartic acid are held tightly in particulate matter of the cell. Although no attempt was made to isolate a pure mitochondrial fraction, it seems, from the method of preparation of the homogenate (3), that essentially all of the "aspartic oxidase" is in the mitochondria. For example, nearly all of the activity of a washed homogenate was retained in the particulate matter from which nuclei and whole cells were removed by low speed centrifugation, and there was no apparent oxidation

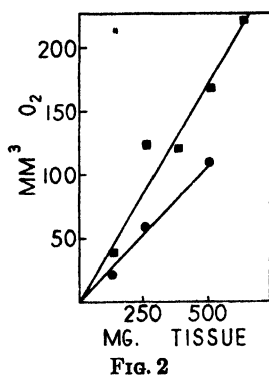


FIG. 2

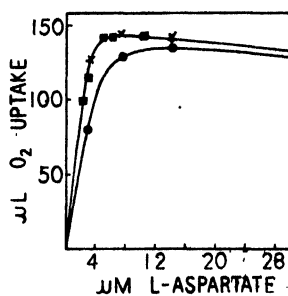


FIG. 3

FIG. 2. Effect of tissue concentration, two separate experiments. Oxygen consumption corrected for endogenous rate.

FIG. 3. Effect of substrate concentration. Abscissa, micromoles of aspartate per 3 ml. of total volume. ●, ■, and × represent separate experiments. Oxygen consumption corrected for endogenous rate.

of aspartate in the supernatant fluid containing smaller particles and soluble matter. The data of Fig. 3 reveal that oxygen uptake is maximal at an aspartate level of 0.002 M, the half saturation concentration being in the neighborhood of 0.0006 M.

Effect of Inhibitors—Since the particulate matter of liver is the site of enzymes responsible for oxidations by way of the citric acid cycle, it could be presumed that the oxidation of aspartic acid was proceeding by this pathway. Further evidence for the participation of the citric acid cycle in aspartate oxidation was obtained by studies of the effects of inhibitors, which are presented in Table II. Freezing of the homogenate, or washing it in distilled water, procedures which are known to destroy the activity of the "cyclophorase system" toward components of the citric acid cycle (4) and which we have found also to inactivate rat liver homogenates for

the oxidation of fatty acids and pyruvic acid, also destroys the aspartate oxidase activity of the rat liver homogenate. Arsenite in 0.01 M concentration, which inhibits oxidation of fatty acids in the cyclophorase system (5), completely inhibited aspartate oxidation. The complete inhibition of aspartate oxidation by malonate obviously cannot be explained on the basis of its effect on succinate oxidation, for the absence of the succinate to fumarate step should not interfere with the partial oxidation of aspartic acid up to succinate. According to Potter *et al.* (6), however, malonate has inhibitory effects on other reactions of the citric acid cycle besides succinate oxidation; it is probable that the oxidation of oxalacetate is inhibited by malonate.

The effect of *trans*-aconitate in inhibiting the oxidation of aspartate is of particular interest inasmuch as the site of its action, according to Saffran

TABLE II
Effect of Inhibitors

The oxygen uptake values are given in microliters.

Inhibitor	No substrate		L-Aspartat	
	Inhibitor present	Inhibitor absent	Inhibitor present	Inhibitor absent
Freezing and thawing	15	45	22	209
Distilled water.	20	70	20	190
Malonate (0.01 M)	40	60	29	189
Arsenite (0.01 ")	30	60	30	145
<i>trans</i> -Aconitate (0.01 M)	60	100	60	180
Ammonium chloride (0.01 M)	5	24	84	144

and Prado (7), is on aconitase. These authors were able to demonstrate partial inhibition of the respiration of various tissues *in vitro* accompanied by accumulation of citrate in the presence of *trans*-aconitate. With aspartate, inhibition by *trans*-aconitate was complete, and no citric acid accumulated.

Transaminase Activity of Washed Rat Liver Homogenates—There are two conceivable points of entry of aspartate carbon into the citric acid cycle: transamination with citrulline to yield arginine and malate, according to the recent findings of Ratner and Pappas (1), and transamination with keto acids such as pyruvic or α -ketoglutaric to yield oxalacetic acid. The former mechanism was excluded from consideration because of the absence of citrulline; as a matter of fact, no appreciable amounts of urea were ever observed. The detection of glutamic acid by paper chromatography of the solutions remaining after aspartate oxidation directed our attention to the aspartic-glutamic transaminase system. Assay of the washed homo-

genate for aspartic-glutamic transaminase by the method of Ames and Elvehjem (8) revealed that a significant portion of this presumably soluble enzyme is retained in the particles in a form not removed by repeated washing (Table III). Glutamic acid was the only amino acid to show up regularly in substantial amounts on paper chromatograms; spots characteristic of glycine and alanine were usually observed, but these were always very faint and did not increase significantly during aspartate oxidation. A quantitative evaluation of the formation of glutamate during aspartate oxidation was obtained by a specific decarboxylase of *Escherichia coli*. As shown in Table IV, the oxidation of aspartic acid invariably resulted in

TABLE III

Transaminase Activity of Rat Liver Fractions

The values are given as $Q_{4^{10}}$ (microliters of CO_2 (from oxalacetate) per mg. of dry weight of original tissue per 10 minutes).

	Whole homogenate	Supernatant	Homogenate washed 3 times
Transaminase activity.	12.7	9.7	2.9

TABLE IV

Glutamate Formation from Aspartate

All values are given in micromoles.

Experiment No.	No substrate		L-Aspartate (0.005 M)	
	Oxygen uptake	Glutamate found	Oxygen uptake	Glutamate found
1	3.9	0.05	12.2	2.33
2	4.1	0.05	15.5	1.85
3	3.8	0.15	6.0	1.25
4	3.7	0.20	5.9	1.40
5	1.8	0.05	11.1	1.75

the formation of glutamic acid, although the amount varied from one experiment to another. Approximately 2.5 to 6 moles of oxygen were utilized for each mole of glutamate formed. As glutamic acid is formed, part of it at least can be expected to undergo oxidation to α -ketoglutarate and ammonia, as a result of the action of glutamic dehydrogenase (9). Attempts were made to determine ammonia, and, although increases were always observed, the results were inconclusive because of a large and variable endogenous production of ammonia, which we could not control sufficiently for an accurate evaluation of the net formation from aspartate.

Oxidation of Aspartate in Tissue Slices—It is rather paradoxical that L-aspartate can be oxidized by the washed liver preparations, whereas

according to Krebs (10), Felix and Naka (11), and Ratner and Pappas (1) this acid does not increase the oxygen consumption of liver slices or whole homogenate, a finding which we have also made in numerous experiments. To determine whether aspartic acid is oxidized by liver slices, several experiments were carried out with isotopic DL-aspartic acid labeled with carbon 13 in the β -carboxyl carbon ($\text{HOOC}^{13}\text{CH}_2\text{CHNH}_2\text{COOH}$). As shown in Table V, both kidney and liver slices can split off the β -carboxyl of aspartic acid to yield CO_2 . While this finding in itself is not rigid proof

TABLE V

Oxidation of C^{13} -Labeled DL-Aspartate by Rat Liver and Kidney

Isotopic aspartate labeled in β -carboxyl carbon with 3.36 atom per cent excess C^{13} . Experiments run 2 hours at 38° with oxygen as gas phase.

	Experiment 1 Liver slices, 4.55 gm.	Experiment 2 Kidney slices, 2.1 gm.	Experiment 3 Liver homogenate, 4.3 gm.
O_2 uptake, μM	770	578	180
Respiratory CO_2 , μM	580	515	128
“ “ C^{13} , atom per cent excess.....	0.24	0.53	0.80

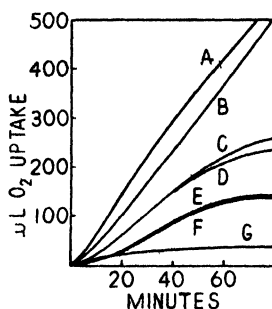


FIG. 4. Oxidation of aspartate by washed rabbit liver homogenate. Curve A, D-aspartate, 0.005 M; Curve B, L-aspartate, 0.005 M; Curves C and D, L- and D-aspartate, 0.001 M; Curves E and F, D- and L-aspartate, 0.0005 M; Curve G, no substrate.

of the oxidation of aspartate, it does indicate that this amino acid is not inert in the tissue slices. The most reasonable interpretation is that the labeled β -carboxyl splits off as CO_2 after conversion to oxalacetate, either by direct decarboxylation to pyruvate or in one of the subsequent decarboxylation steps of the citric acid cycle. As expected, the C^{13} excess of the respiratory CO_2 obtained from the oxidation of aspartate in the washed homogenate was particularly high, a reflection of the absence of endogenous metabolites in this preparation.

Oxidation of Aspartate in Rabbit Liver—Although rat liver homogenates

do not oxidize D-aspartate, we have found, in agreement with the recent observations of Still *et al.* (2), that the unphysiological isomer is readily oxidized by washed homogenates of rabbit liver (Fig. 4). However, we also found, in contrast with these authors, that L-aspartate is also oxidized by the same rabbit liver preparation, and to about the same degree, judging from oxygen uptake, as the D isomer. As shown in Fig. 4, another difference between the rat and rabbit liver homogenate is the remarkable stability of the latter. In contrast with the rat homogenate, which is inactivated after a relatively short period of incubation, the rabbit homogenate continues aspartate oxidation at a linear rate for several hours, or until exhaustion of the substrate.

EXPERIMENTAL

The animals used were male rats, 3 to 6 months of age, weighing about 200 gm. They were killed by decapitation and the livers homogenized by the Potter-Elvehjem technique in isotonic KCl-phosphate solution of pH 7.7. The washed homogenates were prepared essentially according to the procedure of Lehninger and Kennedy (3), except that the centrifugations were carried out with 0.005 M Mg^{++} ions in the medium, since this was found to aid in clumping and separation of the tissue particles. The final concentrations of the components of the medium were as follows: $MgSO_4$, 0.005 M; phosphate buffer, pH 7.7, 0.0125 M; KCl, 0.131 M; ATP, 0.001 M and 10 M KOH in the center well to absorb CO_2 . The total volume was 3 ml. per flask and each flask contained tissue from 500 mg. of fresh liver. Unless otherwise stated, the substrate concentration was 0.005 M L-aspartate. Oxygen uptake was measured by usual Warburg procedures at 38°, with air as the gas phase. All experiments were run for 1 hour unless otherwise stated. Homogenates of rabbit liver were prepared in similar fashion, the animals being killed by air embolism.

Both the D- and L-aspartic acids were obtained from the Nutritional Biochemicals Corporation, and their purity was checked by nitrogen analyses and paper chromatography. The optical rotation of each isomer agreed very closely with the values given for these acids; L-aspartic acid in 6 N HCl, $[\alpha]_D^{22} = +24.6^\circ$; D-aspartic, $[\alpha]_D^{22} = -24.6^\circ$.

The isotopic DL-aspartic acid, having an over-all C^{13} excess of 0.84 atom per cent, was prepared by Strassman (12), essentially according to the procedure of Redemann and Dunn (13), starting with ethyl bromoacetate labeled in the carboxyl position. This was prepared from labeled sodium acetate by the method of Sakami *et al.* (14). C^{13} analyses were made as previously described (15).

Urea was determined by the manometric urease method of Krebs and Henseleit (16), acetoacetate and hydroxybutyrate by the micromethod of

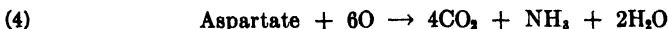
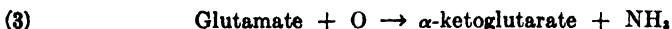
Barnes and Wick (17), keto acids by the method of Friedemann and Haugen (18), and citric acid by the method of Pucher *et al.* (19). Glutamic acid was determined manometrically with a specific L-glutamic acid decarboxylase from *E. coli* 4157, prepared by the method of Umbreit and Gunsalus (20). We are greatly indebted to Dr. R. H. Burris for a culture of this organism and further directions for glutamate determination. Paper chromatography was carried out according to the procedure of Williams and Kirby (21). Transaminase activity was measured by the method of Green *et al.* (22) as modified for homogenates by Ames and Elvehjem (8).

The tissue slice experiments in Table V with isotopic aspartate were carried out exactly as were previous experiments with isotopic fatty acids (23) at a substrate concentration of 0.01 M. The experiments were carried out for 2 hours at 38° with oxygen as the gas phase.

DISCUSSION

It has been shown by Braunstein and coworkers (24) that the properties of the "L-amino acid oxidase" described by Krebs (10) are consistent with the operation of two enzymes: a glutamic transaminase, which transfers nitrogen to α -ketoglutarate, and glutamic dehydrogenase, which liberates ammonia and regenerates α -ketoglutarate for further transamination. The following three points, which represent our experimental findings, are in full accord with this interpretation of the oxidation of L-aspartic acid in washed rat liver homogenate: (1) aspartic-glutamic transaminase is present in preparations which oxidize aspartic acid, (2) aspartate oxidation is inhibited by substances which inhibit the oxidation of oxalacetate or other substances through the citric acid cycle, (3) glutamic acid accumulates during the oxidation of aspartic acid.

The over-all oxidation of aspartate may be represented by the following equations.



Among the complex interplay of reactions leading to the oxidation of aspartic acid, it is evident that one of the main factors controlling the over-all reaction velocity is the rate at which α -ketoglutarate is regenerated. There are at least two main sources of α -ketoglutarate: the citric acid cycle and glutamic dehydrogenation. Inasmuch as the over-all rate of oxalacetate oxidation, which supplies ketoglutarate through the Krebs cycle, is itself mainly dependent on the rate at which oxalacetate is gen-

erated through transamination, the rate of the over-all process must depend ultimately on the rate of regeneration of α -ketoglutarate by way of glutamic dehydrogenation. For these reasons we may anticipate that any factor which affects the rate of glutamic dehydrogenation will also affect aspartate oxidation. It has been found that the oxidation of aspartate falls short of completion, as represented in equation (4), in that a substantial quantity of glutamate accumulates; coincidentally, there is also a rapid falling off in the rate of oxygen uptake after 30 minutes, an effect which could not be attributed to depletion of either substrate or high energy phosphate. The responsible factor in both of these effects is the accumulation of ammonia, which inhibits glutamic dehydrogenation by driving the equilibrium of reaction (3) in the direction of amination, thereby causing an accumulation of glutamate, and at the same time cutting off the supply of α -ketoglutarate required to initiate aspartate oxidation. Support for this idea is found in the fact that the addition of ammonia inhibits aspartate oxidation.

On the basis of the above reasoning, the accumulation of ammonia by autolysis of endogenous proteins may conceivably play a similar part in the generally rapid inactivation of other oxidative reactions in washed homogenates. Such conditions would not be encountered in the normal intact cell in which hormonal or other influences would balance these reactions to the needs of the organism as a whole. In the functioning, whole liver cell, for example, nitrogen can be transferred from aspartate to urea by way of citrulline and arginine (1).

SUMMARY

1. Washed particles of rat liver, when fortified with adenine nucleotides, oxidize L-aspartic acid but not the D isomer. Similar preparations of rabbit liver oxidize both isomers at approximately equal rates.

2. Aspartic-glutamic transaminase is present in the washed liver homogenates and glutamic acid accumulates as a product of aspartate oxidation.

3. The oxidation of aspartate in washed homogenates is inhibited by substances which inhibit reactions of the citric acid cycle.

The foregoing evidence is presented as a basis for the belief that L-aspartate is not oxidized by a specific enzyme, but its oxidation represents the combined action of aspartic-glutamic transaminase, glutamic dehydrogenase, and the reactions of the citric acid cycle.

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CONCENTRATION OF COLLAGEN IN GUINEA PIG TISSUES IN ACUTE AND PROLONGED SCURVY*

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Numerous experimental and clinical observations throughout the years have shown that an ascorbic acid deficiency in man, other primates, and guinea pigs disrupts the normal metabolism of intercellular mesenchymal tissue materials. Wolbach and Bessey (1), in reviewing the tissue changes accompanying vitamin C deficiency, stated, "Specifically this narrows down to problems of tissue collagen formation and its maintenance." The above conclusions have been drawn primarily on the basis of morphologic and histologic criteria and are generally accepted.

As part of a program of study of the biochemical rôle of ascorbic acid in the metabolism of the intercellular matrix, an investigation of the quantitative changes in the collagen in tissue of young guinea pigs subjected to scurvy was undertaken. The tissues examined included some of high and some of low collagen content, some which are obviously subjected to physical stress and some which are not, some which in scorbutic animals show marked histologic changes and some which show less. Tissues from guinea pigs maintained on inadequate amounts of ascorbic acid for periods up to 17 weeks were also studied, since such animals often show greater evidence of connective tissue degeneration than do those dying of acute scurvy.

EXPERIMENTAL

Male guinea pigs from a commercial source were fed a Macdonald (2)¹ scorbutigenic Diet 5 orally supplemented with 5000 units of vitamin A and 800 units of vitamin D (fortified cod liver oil) plus 40 mg. of α -tocopherol, administered as a combined solution once a week, and 50 mg. of ascorbic acid in a 5 per cent sucrose solution every other day. Straw and water were available *ad libitum*. When the animals reached a weight of about 300 to 350 gm., they were placed on experiment.

Acute scurvy was produced by withholding ascorbic acid. The animals

* This work was performed in part under contract N9-onr-82800 with the Office of Naval Research.

¹ Dr. E. W. Crampton of Macdonald College, Quebec, very kindly made available an adequate supply of this diet.

were watched carefully and killed by decapitation when death seemed imminent. Some animals died during the night; these were not used if there was evidence of tissue autolysis.

Prolonged scurvy was produced by withholding ascorbic acid for 2 weeks and then administering 0.1 to 0.35 mg. of ascorbic acid per day so that there was a progressive loss of weight. These animals were killed or died between the 6th and 17th weeks.

Preparation of Tissues—Skin was depilated with a barium sulfide suspension and a girdle about an inch wide was removed from midway between the fore paws and rear legs; loose fascia, fat, and muscle were removed and the skin cut into small pieces.

Livers, lungs, kidneys, and spleens were freed of superficial connective tissue and fat; vessels and ducts were cut away at the hilar region and the tissues placed in weighing bottles and minced.

Costochondral junctions were obtained after dissecting the ribs away from muscle, then cut into pieces 5 to 10 mm. long, consisting of approximately equal lengths of bone and cartilage; these were frozen, dried in a vacuum desiccator to constant weight, defatted with ether and benzene, then ground in a Wiley mill to pass a 40 mesh sieve. The incisor teeth with the immediately adjacent alveolar bone were treated in the same manner.

For each analysis tissues from one animal were used except in the case of kidneys, costochondral junctions, and teeth, which were pooled samples from two animals, and spleens, which were taken from two to five guinea pigs.

Tissue sections were fixed for histologic examination.²

Analytical Methods—Collagen was assayed by the method of Lowry *et al.* (3) with only slight modifications. Tissues were first extracted with water to prevent formation of a very viscous solution (4). Maceration and dispersion of collagenous tissues such as skin with mortar and pestle were accomplished more successfully after they had been softened by treatment with 0.1 N NaOH. The 24 hour extraction with alkali was repeated four or five times.

Total solids were determined by drying an aliquot to constant weight at 105°, and neutral fat by measuring the loss of weight of the dried aliquot after extraction with ether and benzene (5). Blood content of liver, lung, and kidney was estimated by spectrophotometric analysis of a dilute ammonium hydroxide extract of the tissues.³

² It is a pleasure to acknowledge the generous assistance of Dr. E. Woll and Mr. Boldosser of the Department of Pathology.

³ Unpublished.

Results

Deprivation of ascorbic acid produced no obvious symptoms in the guinea pigs for about 2 weeks; neither growth nor activity appeared to be inhibited. After this time the animals became less active; many assumed the classical face ache position; weight fell off markedly. At death, which occurred on the average 28 days after ascorbic acid was withheld, the average weight was 68 per cent of the maximum. At autopsy all of these guinea pigs showed evidences of scurvy in varying degrees; loose teeth, hemorrhagic joints, muscles, and lymph nodes, subcutaneous and intra-peritoneal hemorrhages, and enlargement of the costochondral junctions and of the adrenal glands. The diagnosis of acute scurvy was confirmed

TABLE I
Concentration of Collagen in Guinea Pig Tissues

All the values are given in gm. of collagen per 100 gm. of dry, fat-free, blood-free tissue, except for spleen, which is given as gm. of collagen per 100 gm. of fresh tissue.

The data are presented as the mean plus and minus the standard deviation of the mean; the numbers in parentheses indicate the number of analyses.

Tissue	Normal	Acute	Prolonged scurvy
Skin	63 $\pm$ 1.7 (9)	64 $\pm$ 1.5 (9)	69 $\pm$ 1.7 (9)
Liver	0.89 $\pm$ 0.06 (8)	1.9 $\pm$ 0.25 (8)	1.6 $\pm$ 0.17 (9)
Kidney	2.9 $\pm$ 0.27 (7)	2.4 $\pm$ 0.28 (8)	3.0 $\pm$ 0.22 (9)
Lung	8.6 $\pm$ 0.40 (14)	9.0 $\pm$ 0.46 (10)	11.0 $\pm$ 0.58 (13)
Teeth	17.9 $\pm$ 0.05 (7)	17.0 $\pm$ 0.15 (8)	17.1 $\pm$ 0.12 (9)
Costochondral junction	26.7 $\pm$ 0.33 (8)	26.4 $\pm$ 0.70 (8)	25.1 $\pm$ 0.60 (9)
Spleen	0.55 $\pm$ 0.05 (9)	0.42 $\pm$ 0.04 (12)	0.40 $\pm$ 0.06 (6)

by histologic examination of teeth, costochondral junctions, and visceral organs.

The weight of the guinea pigs, which were maintained with minimal ascorbic acid administered, beginning with the 2nd week showed slight increases or decreases from one weighing period to the next (3 or 4 days); the general trend was down until at death the weight was approximately 70 per cent of the maximal weight. The average survival time was 11 weeks (range 6 to 17). The behavior and appearance of these animals were similar to those of the group with acute scurvy and at autopsy they invariably showed all gross and microscopic signs of scurvy.

The results of the collagen analyses of the three groups are presented in Table I. When not limited by the amount of material available, we have chosen to present the concentrations as gm. of collagen per 100 gm.

of dry, fat-free, blood-free tissue. The over-all picture for any tissue would be unchanged if expressed on the basis of fresh tissue.

From these data it may be concluded that neither acute nor prolonged scurvy markedly decreased the concentration of collagen in the tissues of the young growing guinea pig. As a matter of fact, the collagen concentration in tissues of scorbutic animals was generally somewhat higher than normal. It seems reasonable to ascribe this to the accompanying inanition during which other tissue substances were mobilized to a greater extent than collagen.

The only tissues in which a small but significant⁴ decrease in collagen occurred, teeth and costochondral junctions, are those which not only show the most marked and consistent changes in an ascorbic acid deficiency but also contain smaller amounts of active metabolites.

DISCUSSION

In view of the general impression that ascorbic acid is necessary for the maintenance of collagen, it was surprising to find no marked decrease during scurvy of the collagen concentration of the several tissues studied. The small decrease observed in teeth and alveolar bone should probably not be interpreted as a loss in collagen but as a dilution of already existing collagen, since teeth continue growth even during scurvy, although without formation of collagenous material. It appears, therefore, that ascorbic acid is not essential for maintenance of preformed collagen in tissues of guinea pigs. These findings are similar to those obtained by Lightfoot and Coolidge (7), who found a small decrease in the collagen concentration of the bones of a 10 day-old scorbutic guinea pig but no decrease in the muscles or connective tissue fascia. In animals of the same age as those used in our experiments, these investigators found no decrease in collagen in any of the three tissues. The analyses reported by these coworkers were based on the method of Spencer *et al.* (8) in which even a marked breakdown of collagen, *e.g.* to gelatin, might not show up since total tannic acid-precipitable nitrogen after hydrolysis of the tissue is determined by this method.

We hoped by the use of the Lowry method, which involves extraction with 0.1 N sodium hydroxide before hydrolysis, that any degradation products of collagen would be extracted and even a partial breakdown of collagen would be reflected in a decreased concentration. It has not been possible to examine rigorously the degree of breakdown of collagen which must occur before the collagen becomes alkali-soluble. Although rat tail collagen which had been dispersed in 0.1 per cent acetic acid, a procedure

⁴ The probability that the value of *t* obtained would be exceeded by chance was less than one in twenty (6).

mild enough to permit recovery of collagen fibers, remained dispersed when added to dilute sodium hydroxide, it was our impression that material which is described histologically as reticulum or procollagen would assay as collagen by this method. It is, therefore, possible that subtle changes, perhaps only of the physical state of the collagen micelle, may have occurred in the tissues of scorbutic animals.

A reexamination of the literature on experimental scurvy revealed that, although all investigators have found that ascorbic acid deficiency produced an inability to lay down new collagen, the necessity of ascorbic acid for preserving formed collagen was not as clearly established. In some cases the term "maintenance" has been used to cover the integrity of the entire tissue collagen.

Hunt (9), however, has by histologic techniques demonstrated a reversion of collagen in newly formed scars as the result of scurvy, and the older literature contains references to healed wounds which opened many years later when a person became scorbutic. The scorbutic syndrome itself suggests a breakdown of collagen. It may well be that only recently formed collagen requires ascorbic acid for its maintenance and that this is a very small fraction of the total collagen present in a given organ.

SUMMARY

The concentration of collagen in skin, liver, lung, kidney, spleen, costochondral junctions, and teeth of normal guinea pigs, and of acutely and chronically scorbutic guinea pigs, has been determined.

The collagen concentration of the tissues of scorbutic guinea pigs was in no case appreciably lower than in normal animals. This suggests that if collagen was not maintained either the amount destroyed was small or the degradation of structure was slight.

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FLUOROACETATE INHIBITION OF FATTY ACID OXIDATION BY LIVER PREPARATIONS

A MECHANISM OF ACTION*

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Sodium monofluoroacetate inhibits the oxidation of acetate (1, 2), and, since a 2-carbon compound similar to acetate is also produced during the metabolism of fatty acids (see reviews by Wood (3) and Bloch (4)), it is not surprising that sodium fluoroacetate also inhibits the oxidation of fatty acids (1, 5, 6). In the opinion of Grafflin and Green (6), this effect requires further investigation. Lehninger,¹ however, failed to obtain inhibition of fatty acid oxidation using a washed rat liver preparation in the presence of 0.001 M fluoroacetate.

This work was undertaken to study further the effect on fatty acid oxidase by this inhibitor. It was found that the fatty acid oxidase of rabbit liver is not as sensitive to this inhibitor as the fatty acid oxidase of rabbit kidney. There is a good correlation between the inhibition of oxygen uptake, caproate utilization, and acetoacetate production, the inhibition being reversed by the addition of Mg^{++} . Neither the inhibition of acetate oxidation nor the formation of a fluorocitrate (7-9) can readily explain these results. Another possible mechanism of fluoroacetate activity is therefore proposed; namely, complex formation with magnesium ions.

Methods

Tissue Preparation—A modification of Lehninger's method (10) was employed. Rabbit liver was chilled, ground in a mortar, then mixed with 4 volumes of saline buffer (containing NaCl, KCl, $MgSO_4$, and phosphate buffer, pH 7.5 (11)), homogenized in a loosely fitting glass homogenizer (12) for not more than 2 minutes, and strained through cheese-cloth. 80 ml. of the filtrate were then centrifuged in a cooled high speed centrifuge for 5 minutes at $20,000 \times g$. The supernatant was discarded and the residue made up to approximately 15 ml. with saline buffer.

* Taken in part from a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Biochemistry in the Graduate College of the State University of Iowa, August, 1949. This work was supported by grants from the Nutrition Foundation, Inc., Smith, Kline and French Laboratories, and the Office of Naval Research.

¹ Lehninger, A. L., private communication.

Analytical Methods—The experiments were carried out in a conventional Warburg apparatus. In all cases, 0.8 ml. of the enzyme suspension (which contained approximately 70 mg. dry weight of tissue) plus MgSO_4 , buffer, and water were placed in the main compartment of the 15 ml. flask, while the sodium caproate and fluoroacetate were added from two side arms after temperature equilibration. The total volume of the liquid phase was 2.0 ml. plus 0.3 ml. of 20 per cent KOH, together with folded filter paper in the alkali well. The temperature was 30.2° , the gas phase air, and the time of incubation 2 hours. Acetoacetate was determined according to Edson (13). The residual caproate was determined by the steam distillation method of Friedemann (14) and is deproteinized with an equal volume of 10 per cent metaphosphoric acid. Under the conditions of this method, about 10 per cent of the fluoroacetic acid was steam-distilled. This is in agreement with a similar method used by Liebecq and Peters (15) in which they obtained 9 per cent recovery.

The ionization constant of monofluoroacetic acid was determined by steam distilling 0.5 gm. of sodium monofluoroacetate plus 10 ml. of 10 per cent H_2SO_4 , according to Friedemann (14). After aeration with CO_2 -free air, the pH was determined on the Beckman pH meter and a 15 ml. aliquot was titrated to a phenolphthalein end-point with a 0.0048 M solution of NaOH. From these data the ionization constant was found to be 1.94×10^{-3} and $\text{pK}_a = 2.71$, which closely approaches the values of 2.10×10^{-3} and $\text{pK}_a = 2.68$ quoted by Chenoweth (16).

The association constants of the two magnesium-fluoroacetate complexes were determined by the method of Cannan and Kibrick (17). 2 ml. of 1.00 M KCl were added to 5 ml. of the fluoroacetic acid distillate described above, and the solution was mixed and diluted to 10 ml. In another flask, 1.5 ml. of 1.00 M KCl and 0.5 ml. of 0.2974 M MgCl_2 were added to 5 ml. of the fluoroacetic acid distillate and the solution was diluted to 10 ml. The pH of both solutions was determined, and the apparent H^+ was calculated. Since the junction potentials of the two solutions are approximately the same, the difference between the two apparent hydrogen ion concentrations is due only to the increased ionization of fluoroacetic acid when the magnesium-fluoroacetate complex is formed. Knowing the junction potential of the solution containing Mg^{++} , we calculated the actual H^+ . By using the actual H^+ and the ionization constant of fluoroacetic acid, the concentration of the free fluoroacetate ion, and, subsequently, the concentration of fluoroacetate associated with Mg^{++} were determined by the usual equilibrium formula. From the concentrations of the total magnesium and of the magnesium-fluoroacetate complex, the unassociated magnesium could be determined.

By substituting the appropriate values in the following equations, the

association constants of $\text{Mg}(\text{FAc})^+$ (C_1) and $\text{Mg}(\text{FAc})_2$ (C_2) were determined.

$$C_1 \quad \frac{\text{Mg}(\text{FAc})^+}{(\text{Mg}^{++})(\text{FAc}^-)} = \frac{(7.8 \times 10^{-4})}{(1.407 \times 10^{-3})(1.44 \times 10^{-3})} \quad 38.5$$

$$C_2 \quad \frac{\text{Mg}(\text{FAc})_2}{(\text{Mg}^{++})(\text{FAc}^-)^2} = \frac{(3.9 \times 10^{-4})}{(0.01448)(1.44 \times 10^{-3})^2} \quad 1.3 \times 10^4$$

Results

One of the difficulties with studies concerning the metabolism of fatty acids arises from the fact that fatty acids in high concentrations actually inhibit the enzymatic reaction. This is probably due to the fact that

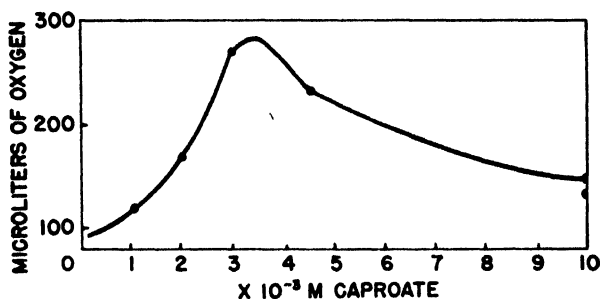


FIG. 1. The relationship of the caproate concentration to the oxygen uptake. Conditions as in Table I; no fluoroacetate present; caproate in indicated concentrations.

fatty acids reduce the surface tension to such an extent that it interferes with the normal oxidase system (18). With various concentrations of caproate, the optimum concentration was found to be 0.003 M, as is shown from the data in Fig. 1.

This is in agreement with Lehninger's finding (18) that the enzyme system was saturated at a concentration of 0.0025 M octanoate. In all subsequent experiments this concentration (0.003 M) was used.

With rabbit kidney cortex preparations, a concentration of 0.001 M fluoroacetate produces complete inhibition of caproate oxidation (5). With the rabbit liver preparations used in these experiments, 0.001 M fluoroacetate produced only a negligible inhibition (Table I). Indeed, complete inhibition with the liver preparation did not occur until concentrations greater than 0.01 M fluoroacetate were used (Table I). This shows that the fatty acid oxidase system in rabbit liver preparations is approximately one-tenth to one-fifteenth as sensitive to fluoroacetate as a similar system in rabbit kidney cortex.

Analysis of acetoacetate produced showed that the formation of this keto acid from caproate was inhibited to about the same extent as the oxygen uptake at various concentrations of fluoroacetate (Table I). In a similar analysis of the caproate utilized by the liver suspension in the presence of 5×10^{-3} M fluoroacetate, it was found that caproate utilization and acetoacetate formation were inhibited to about the same extent as that of the oxygen uptake (Table II). Therefore, the oxygen uptake is a true criterion of the amount of fatty acid actually metabolized by the liver suspension.

In the absence of fluoroacetate, approximately 1 μ M of acetoacetate was formed and approximately 4 μ M of oxygen respired for every micromole of

TABLE I

Effect of Various Concentrations of Sodium Fluoroacetate on Caproate Oxidation and Acetoacetate Formation

Fluoroacetate concentration	O ₂ uptake	Inhibition	Acetoacetate formed	Inhibition
M	μ l.	per cent	μ l.	per cent
	514		93	0
1×10^{-4}	477	7	97	0
1×10^{-3}	495	4	102	0
5×10^{-3}	149	71	51	45
1×10^{-2}	23	96	7	93

Liver suspension 0.8 ml., caproate 0.003 M, MgSO₄ 7.5×10^{-4} M, phosphate buffer, pH 7.5, 0.02 M; total volume 2.0 ml. + KOH in the center well. Time, 2 hours; atmosphere, air; temperature, 30.2°.

caproate utilized (Table II). This would indicate that the following reaction was taking place, under the conditions of these experiments.



In the presence of fluoroacetate, approximately the same ratio of caproate to oxygen to acetoacetate was observed (Table II).

Fig. 2 shows that the inhibition due to the fluoroacetate does not begin immediately but occurs only after an incubation period of about 30 minutes. This induction period may be similar to that found *in vivo* (16) or when pyruvate or citrate was metabolized *in vitro* (5). As has been shown by Bartlett and Barron (1), fluoroacetate does not inhibit the formation of acetoacetate from acetate but actually increases it. If the inhibition of the fatty acid occurred at the level of the 2-carbon intermediate, therefore, the formation of acetoacetate would not be inhibited. Since the formation of acetoacetate from caproate is inhibited to about the same de-

gree as the oxygen taken up and the caproate utilized, the mechanism for the inhibition probably does not occur at the 2-carbon intermediate stage, but at the point of primary oxidation of the fatty acid to the 2-carbon intermediate. It was also shown by Lehninger (10, 18) that the enzyme

TABLE II

Analysis of Acetoacetate Formed and Caproate Utilized and Their Relationship to Oxygen Uptake

Analysis	Experimental conditions		Inhibition <i>per cent</i>
	Caproate μM	Caproate + fluoroacetate μM	
O ₂ uptake.	16.2	5.5	66
Acetoacetate formed.	4.92	1.12	77
O ₂ uptake*.	12.9	1.9	85
Caproate* utilized.	2.72	0.7	74

Same conditions as in Table I.

* Average of two flasks.

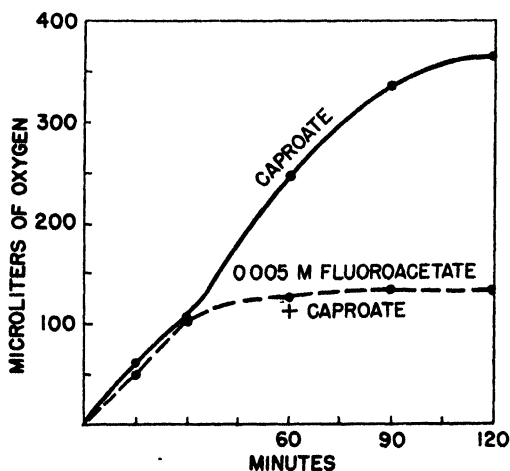


FIG. 2. The effect of 0.005 M sodium fluoroacetate on the rate of oxygen uptake. Same conditions as in Table I.

which oxidizes fatty acids does not oxidize acetate. It seems apparent, then, that the inhibition of caproate oxidation by fluoroacetate in this liver system cannot be attributed to the competitive inhibition mechanism directly.

Leloir and Muñoz (19) and Graffin and Green (6) have demonstrated the necessity of the magnesium ion for the oxidation of various fatty

acids. In the study of this effect, the liver suspension was prepared with a saline buffer which contained no magnesium. Various amounts of magnesium sulfate were added to the Warburg flask before the addition of the enzyme suspension. The results presented in Fig. 3 indicate that the enzyme is inhibited by increased concentrations of magnesium.

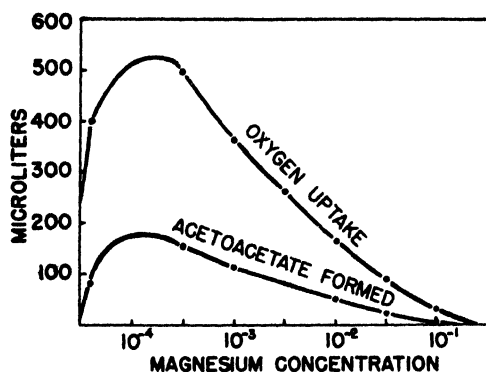


FIG. 3. The effect of magnesium ion concentration on fatty acid oxidation. Conditions as in Table I; no fluoroacetate; magnesium in indicated concentrations.

TABLE III

Effect of Magnesium in Overcoming Inhibition Due to Fluoroacetate

Magnesium	O ₂ uptake		Inhibition	Acetoacetate formed		Inhibition
	Caproate	Caproate + 0.005 M fluoroacetate		Caproate	Caproate + 0.005 M fluoroacetate	
M	μl.	μl.	per cent	μl.	μl.	per cent
5×10^{-4}	500	105	79	154		
7.5×10^{-4}	425	150	65	133	51	62
1×10^{-3}	363	180	50	112		
3×10^{-3}	300	339	0	107	104	3
5×10^{-3}	261	299	0	98	100	0
2×10^{-2}	63	75	0			

In the presence of fluoroacetate there is considerable inhibition of the fatty acid oxidase at low concentrations of magnesium. However, with increasing concentrations of magnesium (particularly at 0.003 M) this inhibition is overcome (Table III).

DISCUSSION

The effect of various concentrations of magnesium on the inhibition by fluoroacetate is especially significant when one considers the work of

Pardee and Potter (20). In their work, malonate was demonstrated to inhibit the oxidation of oxalacetate by forming a complex with magnesium, indicating a second mechanism for the inhibition with malonate. By the use of the method of Cannan and Kibrick (17), we were able to demonstrate that a similar complex exists between fluoroacetate and magnesium.

In order to find out whether the association constants of either $\text{Mg}(\text{FAC})^+$ or $\text{Mg}(\text{FAC})_2$ were of the right magnitude to explain the observations, the equation derived by Pardee and Potter (20) was employed,

$$v = \frac{VK_1\text{Mg}}{(1 + (1 + C\text{FAC})K_1\text{Mg})(1 + K_2\text{Mg})} \quad (2)$$

in which v is the velocity of the reaction and Mg and FAC are the concentrations of the *ionized* magnesium and fluoroacetate. The constants V , K_1 , and K_2 were determined from the points on the oxygen uptake curve of Fig. 3 and were found to be 900, 4×10^3 , and 430 respectively. The constant C is the association constant of either $\text{Mg}(\text{FAC})^+$ or $\text{Mg}(\text{FAC})_2$, with values of 38.5 and 1.3×10^4 respectively.

At magnesium concentrations of 3 and 5×10^{-3} M, the calculated inhibitions due to $\text{Mg}(\text{FAC})^+$ and $\text{Mg}(\text{FAC})_2$ were 6 to 10 per cent, while the observed inhibition (Table III) was zero. At 1×10^{-3} M magnesium, the calculated inhibitions for $\text{Mg}(\text{FAC})^+$ and $\text{Mg}(\text{FAC})_2$ were 13 and 21 per cent respectively, while the observed inhibition was 50 per cent (Table III). Therefore, the experimental evidence and theoretical calculations indicate that sodium fluoroacetate can combine with magnesium to form a stable complex which is capable of inhibiting fatty acid oxidation by lowering the magnesium concentration. Since complex formation does not *entirely* explain the degree of inhibition due to fluoroacetate at lower concentrations of magnesium, an additional mechanism of inhibition, such as the formation of a fluorocitrate (7-9) and other fluoro derivatives, is still possible.

SUMMARY

The effect of monofluoroacetic acid on fatty acid oxidation by a washed rabbit liver suspension has been studied. It was found that this preparation was not as sensitive to monofluoroacetate as a rabbit kidney cortex suspension. The oxygen uptake, caproate utilization, and acetoacetate formation were all inhibited to approximately the same extent at higher concentrations of fluoroacetate. The point at which this compound inhibits the fatty acid oxidase system has been postulated.

The effect of magnesium on the activity of this suspension has been studied, as well as the effect on the inhibition produced by the monofluoroacetate at various concentrations of magnesium. It has been found that at high concentrations of magnesium the fatty acid oxidase system is in-

hibited. The inhibition due to fluoroacetate, however, is overcome by increasing the magnesium concentration. An attempt has been made to explain these results by the formation of a magnesium-fluoroacetate complex.

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RÔLE OF ALIMENTARY MICROORGANISMS IN THE SYNTHESIS OF NON-ESSENTIAL AMINO ACIDS*

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Recognition of the fact that only ten of the amino acids known to be present in proteins are essential dietary components for the growing rat (1) suggests a number of questions concerning the origin of the non-essential group. That these are made available to the cells, even when absent from the food, is clear from the sustained ability of the subjects to gain in weight. It is axiomatic that tissues either form typical proteins, containing all of the amino acids characteristically and normally present, or form none at all (*cf.* (2)). This attribute of growth endows it with extraordinary biochemical significance as a measure of the extent and rate of synthetic reactions occurring in the organism.

Much progress has been made in recent years in establishing the origin of the non-essential amino acids. Two appear to have specific precursors. These are tyrosine, which is formed from phenylalanine (3), and cystine, which arises from methionine and serine (4-5). Certain others, notably alanine, aspartic acid, and glutamic acid, are produced by the amination or transamination of the corresponding α -keto acids (6), which in turn are liberated in carbohydrate metabolism. Furthermore, certain interconversions are now acknowledged such as those which occur between arginine, the prolines, and probably glutamic acid (7-10), and between glycine and serine (11-12). With respect to the nitrogen of the dispensable amino acids, investigations in this laboratory have revealed that ammonium salts, and even urea, are acceptable sources of this element for the manufacturing purposes of the organism (13).

Synthetic reactions or those of interconversion involving amino acids, when observed in *intact* animals, are frequently difficult to interpret because of the possibility that they may have been accomplished by microorganisms in the alimentary tract rather than by the cells of the host. Indeed, one may well ask whether the growth of animals which have been

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† The experimental data in this paper are taken from a thesis submitted by Leonard C. Smith in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the University of Illinois.

deprived of one or more amino acids may not be due, at least in part, to the activity of the intestinal flora or fauna in manufacturing the missing dietary components. Such a question is pertinent also with regard to the experiments of Rose, Smith, Womack, and Shane (13), in which the addition of any one of several sources of nitrogen to diets containing the minimum levels of the essential amino acids resulted in a pronounced acceleration in the rates at which the subjects gained in weight. Was the ammonium salt or urea used by the microorganisms for the generation of amino acids and were the latter responsible for the more rapid growth?

The above questions are extraordinarily difficult to answer categorically. Before an unequivocal decision can be reached regarding them, the use of germ-free animals may be necessary (*cf.* (14)). However, it appears that some indication of the rôle of microorganisms in these reactions might be obtained by applying the technique employed so successfully in demonstrating the bacterial synthesis of vitamins (15); namely, the inclusion in the food of a slowly absorbed sulfonamide. Of the latter, one of the most widely used for the purpose in question is succinylsulfathiazole (sulfasuxidine). Apparently, the bacteriostatic action of this and other sulfonamides is confined largely to the coliform and lactobacillus groups (15-16), and consequently one or both of these are believed to be responsible for the synthesis of vitamins (17). However, if amino acids are formed in the alimentary tract, it does not follow necessarily that the same organisms are the active agents. Obviously, positive results in experiments of this nature would be of greater significance than negative findings.

A search of the literature reveals a remarkable paucity of data concerning the effects of sulfonamides upon the alimentary synthesis of amino acids. Martin (18) compared the growth of rats upon three diets containing respectively whole casein, an enzymatic digest of casein, and a mixture of the ten essential amino acids. Each diet was administered with and without the inclusion of 2 per cent of sulfasuxidine. The results were inconclusive inasmuch as all the animals for which actual gains are shown were deprived of folic acid. Under the circumstances, it is not surprising that growth was inhibited by the drug even in the subjects which received the casein ration. Since the animals upon the amino acid diet made the poorest showing, the author concludes that the findings "are suggestive of inhibition of amino acid synthesis by bacteria in the intestine of the rat." Somewhat later, Williams and Watson (19) administered an arginine-free ration to six rats, three of which received 2 per cent of sulfasuxidine admixed with the food. Folic acid was not furnished. Since the animals which ingested the drug failed to show a dramatic loss of weight, such as is known to accompany acute amino acid deficiencies, the authors

conclude that the intestinal tract is not the source of the arginine synthesized by this species.

In an attempt to obtain more convincing data regarding the influence of sulfasuxidine upon the growth of animals ingesting amino acid diets, the experiments described in the following pages were undertaken. For this purpose, three diets were formulated containing respectively nineteen amino acids, ten amino acids in amounts sufficient to furnish an adequate nitrogen intake, and ten amino acids at their minimum levels (20, 13) supplemented with diammonium citrate as a source of nitrogen. Each diet was administered to two groups of animals, one of which received sulfasuxidine. It was assumed that the growth rates of the subjects which ingested nineteen amino acids would not be inhibited by the presence of the drug, provided that abundant supplies of vitamins were included in the food and provided further that the drug, at the level employed, exerts no inherent toxic effect upon the animals. On the other hand, the subjects which received either of the diets containing ten amino acids, in addition to the drug, might be expected to gain at slower rates than their controls, provided that the alimentary microorganisms affected by sulfasuxidine are actually involved in the synthesis of the non-essential amino acids. The results of the experiments are presented with full appreciation of the limitations inherent in the method.

EXPERIMENTAL

Throughout the investigation, male weanling rats served as the subjects. Each animal was housed in a separate cage and was permitted to consume food and water *ad libitum*. All tests were continued for 28 days. The amino acids which furnished the nitrogen of the rations were purified invariably until they yielded correct analytical values.

In Table I is presented the composition of three amino acid mixtures which were used in the tests. Mixture XXII contained all of the amino acids known to be present in proteins except citrulline. This mixture is identical in composition with Mixture XXIII (1), which is now regarded as our standard, except that the former contains twice as much leucine and lysine. The absence of citrulline is without effect upon the rate of gain. Furthermore, this amino acid can be synthesized in the liver of the rat (21). Mixture X has not been described previously. It contained the ten essentials only, each in considerable excess over the minimum needs, so as to furnish an adequate nitrogen supply for the synthesis of the non-essentials. Mixture XXVI was discussed in the paper of Rose *et al.* (13). Its composition is shown in Table I merely to facilitate a comparison between it and the other mixtures used in the present experiments.

It furnished each component at its *tentative* minimum level (*cf.* (20) and (13)).

In Table II is shown the make-up of the three diets, containing respectively nineteen amino acids (Diet 1), ten amino acids in excess (Diet 2), and ten amino acids at their minimum levels plus diammonium citrate

TABLE I
Composition of Amino Acid Mixtures

	Mixture XXII		Mixture X		Mixture XXVI	
	Physiologically active	As used	Physiologically active	As used	Physiologically active	As used
	gm.	gm.	gm.	gm.	gm.	gm.
Glycine.....	0.1	0.10				
Alanine.....	0.2	0.40*				
Serine.....	0.1	0.20*				
Valine.....	1.0	2.00*	1.5	3.00*	0.7	1.40*
Leucine.....	2.4	2.40	1.5	1.50	0.8	0.80
Isoleucine.....	0.8	1.60*	1.5	3.00*	0.5	1.00*
Cystine.....	0.2	0.20				
Methionine.....	0.8	0.80*	1.5	1.50*	0.6	0.60*
Threonine.....	0.7	1.40*	0.8	1.60*	0.5	1.00*
Phenylalanine.....	1.2	1.20*	1.5	1.50*	0.9	0.90*
Tyrosine.....	0.6	0.60				
Proline.....	0.2	0.20				
Hydroxyproline.....	0.1	0.10				
Tryptophan.....	0.4	0.40	0.4	0.40	0.2	0.20
Aspartic acid.....	0.2	0.20				
Glutamic ".....	2.0	2.00				
Lysine.....	2.4		1.5		1.0	
" monohydrochloride.....		3.00		1.88		1.25
Histidine.....	0.7		0.6		0.4	
" monohydrochloride monohydrate.....		0.95		0.81		0.54
Arginine.....	0.4		0.4		0.2	
" monohydrochloride.....		0.50		0.50		0.24
Sodium bicarbonate....		1.96		1.39		0.89
	14.5	20.21	11.2	17.08	5.8	8.82

* Racemic acids.

(Diet 3). The diammonium citrate furnished 0.574 gm. of nitrogen per 100 gm. of food. This amount was found previously to support almost twice as rapid growth as a similar ration without an additional source of nitrogen (13). Each diet was appropriately supplemented with vitamins. For this purpose, the quantities listed in Table III were thoroughly admixed with each kilo of food. Preliminary experiments with diets con-

taining casein had shown that the vitamin supplements ordinarily used by us (*cf.* (1)) were insufficient to prevent growth inhibition when sulfasuxidine was incorporated in the food. For this reason, the thiamine, pyridoxine, and nicotinic acid were increased and folic acid was added. The resulting mixture (Table III) prevented a significant difference in the growth rate when the sulfonamide was administered in a casein ration.

When sulfasuxidine was included in Diets 1, 2, or 3, it replaced an equal weight of dextrin. The level of intake was 1 per cent. This is twice the

TABLE II
Composition of Diets

	Diet 1	Diet 2	Diet 3
	gm.	gm.	gm.
Amino acid Mixture XXII.....	20.21		
“ “ “ X.....		17.08	
“ “ “ XXVI.			8.82
Diammonium citrate			4.67*
Sucrose *...	15.00	15.00	15.00
Dextrin	56.04	59.17	62.76
Cellu flour....	2.00	2.00	2.00
Salt mixture†.....	4.00	4.00	4.00
Corn oil.....	2.00	2.00	2.00
Vitamin A and D concentrate‡	0.05	0.05	0.05
Inositol	0.10	0.10	0.10
Choline chloride...	0.20	0.20	0.20
Liver extract§.....	0.40	0.40	0.40
	100.00	100.00	100.00

* Equivalent, after correcting for moisture, to 0.574 gm. of nitrogen per 100 gm. of food.

† Jones and Foster (22).

‡ Contained 65000 U. S. P. units of vitamin A and 13000 U. S. P. units of vitamin D per gm.

§ Wilson's liver powder, 1:20.

level used by Gant *et al.* (17) in demonstrating the effects of the drug upon the intestinal flora of the rat.

Three series of experiments were conducted, the results of which are summarized in Table IV. In Series I, thirty-two male rats from eight litters were divided into two groups containing the same number of animals from each litter. One group received Diet 1 containing nineteen amino acids (Mixture XXII); the other, the same diet, except for the inclusion of 1 per cent of sulfasuxidine. The data in Table IV show that no significant difference occurred in the rates of gain. This was to be expected, as predicted above, inasmuch as all amino acids (except citrul-

line) were present in the food. Under these circumstances, and since the vitamin supplements had already been shown to be adequate, the animals were not dependent for their metabolic needs upon the activity of alimentary microorganisms. The data indicate also that sulfasuxidine, under the conditions of the tests and in the amounts used, does not exert a detectable toxic effect upon the rat.

TABLE III
Vitamin Supplements

	Added to each kilo of diet
	mg.
Thiamine hydrochloride	20
Riboflavin	10
Pyridoxine hydrochloride	10
Nicotinic acid. . .	50
Calcium <i>d</i> -pantothenate	25
<i>p</i> -Aminobenzoic acid	300
α -Tocopherol	25
2-Methyl-1,4-naphthoquinone	2
Biotin.	0.1
Folic acid.	0.3

TABLE IV
Effect of 1 Per Cent Sulfasuxidine on Growth

The experiments covered 28 days each

Series No.	No. of animals (males)	Mean gain in weight and probable error of mean	Presence or absence of sulfasuxidine	Nature of diet
		gm.		
I	16	97.0 $\pm$ 1.61	—	Diet 1, 19 amino acids
	16	95.7 $\pm$ 1.71	+	
II	22	72.1 $\pm$ 1.36	—	" 2, 10 " " in excess
	22	64.8 $\pm$ 1.05	+	
III	15	89.9 $\pm$ 1.33	—	" 3, 10 amino acids at minimum
	13	86.5 $\pm$ 1.32	+	levels + diammonium citrate*

* The diammonium citrate furnished 0.574 gm. of nitrogen per 100 gm. of food.

Having satisfied ourselves that growth is not inhibited by the presence of 1 per cent of the drug, when the ration furnishes nineteen amino acids and an adequate complement of vitamins, we next undertook the crucial tests with mixtures of ten amino acids. In Series II, forty-four males from ten litters were divided into two groups as in the tests of Series I. One group received Diet 2 containing the ten essentials in excess amounts

(Mixture X), and the other group the same diet plus 1 per cent of sulfasuxidine. As will be observed from Table IV, the animals which ingested Diet 2 alone manifested a mean gain of 72.1 ± 1.36 gm. Rose, Oesterling, and Womack (1) have shown that a mixture of the ten essential amino acids is distinctly inferior in nutritive quality to one carrying nineteen amino acids. In their experiments, the mean gain of 50 rats on ten amino acids was found to be 78.5 gm. The difference between the latter figure and the one for the controls in the present study (72.1 gm.), though small, may be due to the fact that Mixture X contained a somewhat higher content of DL-methionine than the comparable mixture (Mixture XXIV) employed earlier (1). Wretling (23) and Van Pilsum and Berg (24) have reported that DL-methionine is the most poorly tolerated of the racemic essential amino acids. However, any effect which may have been exerted by an excess of this amino acid in the current investigation is believed to be inconsequential inasmuch as the control and experimental groups of animals received identical amounts.

Of particular interest is the observation that the subjects upon Diet 2 plus sulfasuxidine showed a mean gain of 64.8 ± 1.05 gm. The difference between this and the mean gain of the controls is 7.3 gm., and the ratio of the mean difference to the probable error of the difference is 4.24. This implies that the odds against the difference being due to chance alone are approximately 240 to 1. Ratios greater than 100 to 1 are commonly considered to be statistically significant. Therefore, on the basis of the above findings, one is forced to assume as a working hypothesis that the microorganisms which are inhibited by sulfasuxidine *may* play a rôle in the synthesis of the non-essential amino acids. However, this rôle must be of minor importance in view of the relatively small difference in the mean gains of the two groups of animals. Either microorganisms which are not impeded by the sulfonamide, or the cells of the host must be largely responsible for the synthesis of non-essential amino acids. If this were not the case, the subjects which received the drug would have *lost* weight instead of gaining at *slightly diminished* rates. The dominant rôle played by microorganisms in the formation of biotin, folic acid, and other vitamins (*cf.* (25)) tends by comparison to minimize the extremely moderate effects observed in the present investigation.

Further evidence of like import is found in the tests of Series III. In the latter, twenty-eight animals from eight litters were distributed between Diet 3 alone and Diet 3 plus 1 per cent of sulfasuxidine. It should be recalled that the amino acid mixture (Mixture XXVI) used in this diet furnished the ten essentials at their *tentative* minimum levels, and that these are capable of supporting only very slow growth unless supplemented with some additional source of nitrogen. In the present study,

the extra nitrogen was supplied in the form of diammonium citrate. Such a ration would appear to afford ideal conditions for a demonstration of the activity, if any, of intestinal microorganisms in the manufacture of amino acids. The results (Table IV) show that the mean gain in weight of the fifteen animals which did not receive sulfasuxidine was 89.9 ± 1.33 gm., while that of their thirteen litter mates which ingested the drug was 86.5 ± 1.32 gm. Thus, the mean difference is 3.4 gm., and the ratio of the mean difference to the probable error of the difference is 1.8. This ratio is certainly not statistically significant. Consequently, one may conclude with considerable assurance that the microorganisms which are inhibited by sulfasuxidine are not involved to a demonstrable extent in the utilization of ammonium salts for the formation of non-essential amino acids. Incidentally, the mean gains of the two groups of animals of Series III are in close agreement with the mean gain (85.9 gm.) of 56 subjects upon a like diet reported in an earlier paper (13). Furthermore, Mixture XXVI, when supplemented with an ammonium salt, invariably appears to afford better growth than an unsupplemented mixture composed of excess quantities of the ten essentials (cf. (1) and (13)).

The above experiments appear to demonstrate that the coliform and lactobacillus groups of bacteria are of much less significance in the synthesis of amino acids than in the manufacture of vitamins. The ultimate solution of the problem must await the discovery of more effective measures for the control of the alimentary flora, or must rely upon the use of animals which have been reared under sterile conditions.

SUMMARY

A large number of experiments have been conducted in order to determine the possible rôle of alimentary microorganisms in the synthesis of the non-essential amino acids. For this purpose, the growth of male weanling rats upon each of three diets has been compared with the behavior of their litter mates upon diets of identical composition except for the inclusion of 1 per cent of sulfasuxidine. The diets contained, respectively, nineteen amino acids, the ten essential amino acids in sufficient amounts to furnish an adequate nitrogen intake, and the ten essentials at their *tentative* minimum levels supplemented with diammonium citrate as a source of nitrogen. Each ration carried an appropriate complement of vitamins.

The inclusion in the food of sulfasuxidine induced no significant inhibition in the growth of the animals which received nineteen amino acids. With the ration containing the ten essentials in moderate excess, the presence of the drug led to a slight, but statistically significant, decrease in the rate of gain. On the contrary, the sulfonamide was without effect upon

the growth of the subjects which consumed the ration containing ten amino acids at their *tentative* minimum levels plus nitrogen in the form of diammonium citrate.

The results do not exclude the possibility that microorganisms which are *not* affected by sulfasuxidine *may* participate in the synthesis of non-essential amino acids. However, the data appear to provide rather convincing proof that the coliform and lactobacillus groups are involved in these reactions to a very limited extent, if at all.

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METHIONINE REQUIREMENT FOR GROWTH AND UTILIZATION OF ITS OPTICAL ISOMERS*

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Several years ago experiments were undertaken in this laboratory for the purpose of determining the minimum amount of each indispensable amino acid which must be included in the food in order to induce maximum growth in the rat. This was accomplished by varying the proportion of one amino acid while maintaining the concentrations of all others at constant levels. Throughout the tests, liberal quantities of the non-essential amino acids were furnished except in the experiments involving methionine and phenylalanine. With these, the rations were devoid of cystine and tyrosine respectively, inasmuch as it had already become evident that the former can arise *in vivo* from methionine, and the latter from phenylalanine. Thus, the values representing the minimum needs of the organism for methionine and phenylalanine really included the quantities of these two amino acids which were used in the manufacture of cystine and tyrosine.

The results of these tests were summarized by one of us in 1937 (1). At the time, attention was called to the fact that the values were "to be regarded as *strictly tentative*." Furthermore, it was pointed out that "factors such as the proportion of fat and carbohydrate in the ration, and the age, weight and sex of the subjects may play important rôles in determining the minimum level of a given component."

The experimental data upon which these tentative values were based were never published in full. After the tests had been completed, systematic investigations extending over several years revealed the necessity of altering our basal diet in order to provide a more appropriate (lower) fat content, a better source of inorganic salts, and a more adequate supply of vitamins (*cf.* Borman *et al.* (2)). All of these modifications permitted the subjects to gain at more rapid rates. It is well known that a ration which furnishes all the essential components is consumed by animals until their caloric requirements have been satisfied. It seemed probable, therefore,

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that a ration of low fat content, which would be consumed in liberal amounts, might permit the use of a smaller *percentage* of a given amino acid without diminishing the rate of gain, than a diet of high fat content which would be ingested more sparingly. On the other hand, the possibility cannot be overlooked that the acceleration in the rate of growth resulting from any improvement in the quality of a basal ration might necessitate an *increase* in the percentage of an essential amino acid in order to support the more rapid synthetic reactions occurring in the cells.

Because of the above considerations, a reinvestigation of the minimum requirements of the growing rat for several amino acids has been undertaken. Elsewhere we have reported that the L-phenylalanine requirement, under the conditions of our tests, is approximately 0.9 per cent of the diet (3). This represents an upward revision of our original figure of 0.7 per cent (1). Evidence has also been presented to the effect that, within the limits of accuracy of the method, D- and L-phenylalanine are essentially equal in nutritive value for growth purposes (3) and that approximately half of the phenylalanine can be replaced by tyrosine (4). Although these values were established at a time when our basal diet had been revised only partially, and still contained a relatively high proportion of fat, the figures are believed to be as accurate as can be expected in tests of this general nature.

The present paper is concerned with a redetermination of the minimum requirement of the growing rat for methionine and the relative nutritive value of the D and L enantiomorphs. In the original experiments cited above (1), which involved the use of a diet that was devoid of cystine, 0.6 per cent of methionine was found to be necessary for maximum gains. The two isomeric forms were regarded as essentially equivalent for growth purposes (5). Furthermore, only one-sixth of the minimum level, or approximately 0.1 per cent, was replaceable by cystine (6).

In the meantime, another factor affecting the methionine content of the food has been revealed; namely, the unique toxicity of this amino acid when it is furnished in moderately excessive amounts. Independently, Wretling (7) and Van Pilsum and Berg (8) have shown that, of the racemic forms of the essential amino acids, DL-methionine is the most poorly tolerated. The presence in the food of 2 to 2.4 per cent induced invariably a retardation in the growth rate. Even more amazing is the fact observed in both laboratories (8, 9) that L-methionine is more deleterious than the D isomer. In view of these unprecedented findings, it becomes necessary, in the case of methionine, to establish not only the minimum level of intake which is capable of supporting maximum gains but also the level beyond which growth inhibition is manifested. The results of such experiments are presented below. Throughout the investigation the two optically active modifications, rather than the racemic amino acid, were used.

EXPERIMENTAL

The D- and L-methionine employed in these studies were prepared by the enzymatic resolution of DL-methionine according to the procedure of Brenner and Kocher (10) as modified by Wrettlind (9). The resulting products yielded the following analytical data.

L-Methionine ($C_5H_{11}O_2NS$). Calculated, N 9.39; found, N 9.40
 $\alpha_D^{25} = +20.3^\circ$ (1.0% in 0.2 N hydrochloric acid)

According to Price, Gilbert, and Greenstein (11), L-methionine has a specific rotation of $+21.6^\circ$ (0.88 per cent in 0.2 N hydrochloric acid).

D-Methionine ($C_5H_{11}O_2NS$). Calculated, N 9.39; found, N 9.42
 $\alpha_D^{25} = -20.0^\circ$ (1.0% in 0.2 N hydrochloric acid)

Dekker and Fruton (12) report a specific rotation of -21.5° (1.2 per cent in 0.2 N hydrochloric acid). An identical value was observed by Price *et al.* (11). Obviously, our products may have been contaminated very slightly with their enantiomorphs, but, if so, the amounts present were far too small to have affected the outcome of the tests.

Male weanling rats having initial weights of 50 to 60 gm. served as the subjects. Each animal was housed in a separate cage and was permitted to consume food and water *ad libitum*. All tests were continued for 28 days. The amino acids which furnished the nitrogen of the rations were purified invariably until they yielded correct analytical values.

In Table I is presented the composition of the basal diet. The amino acid mixture (Mixture XXIII-C) contained all of the amino acids known to be present in proteins except methionine and citrulline. The presence or absence of citrulline does not affect the growth of the rat. Mixture XXIII-C was identical in composition with Mixture XXIII (*cf.* Rose, Oesterling, and Womack (14)), which has been used extensively in this laboratory and is regarded as our standard, except that the former was devoid of methionine and furnished 0.2 gm. of L-aspartic acid instead of 0.4 gm. of DL-aspartic acid per 100 gm. of food. The substitution of L-aspartic acid for the racemic compound is without nutritive significance. Either may be used with equal success. The desired quantities of L- or D-methionine were added to the basal diet at the expense of the dextrin. All the diets were appropriately supplemented with vitamins. For this purpose, the quantities listed in Table II were thoroughly admixed with each kilo of ration.

Attention is called to the fact that in the present experiments the rations uniformly contained 0.2 per cent of L-cystine. This seemed advisable inasmuch as one of the objectives of the tests was to establish the *maximum* level of methionine which is capable of permitting optimum

growth under conditions which otherwise were as near normal as possible. Since cystine is present in stock rations, it was included in the experimental diets. On the other hand, the results obtained under these circumstances

TABLE I
Composition of Basal Diet

	<i>gm.</i>
Amino acid Mixture XXIII-C	16.02
Sucrose	15.00
Dextrin..	60.23
Cellu flour	2.00
Salt mixture*	4.00
Corn oil ..	2.00
Vitamin A and D concentrate†	0.05
Inositol ..	0.10
Choline chloride ..	0.20
Liver extract‡... ..	0.40
	100.00

* Jones and Foster (13).

† This contained 65,000 U. S. P. units of vitamin A and 13,000 U. S. P. units of vitamin D per gm.

‡ Wilson's liver powder, 1:20.

TABLE II
Vitamin Supplements

	<i>mg.</i>
Thiamine hydrochloride . . .	5
Riboflavin	10
Pyridoxine hydrochloride . . .	5
Nicotinic acid	5
Calcium <i>d</i> -pantothenate .	25
<i>p</i> -Aminobenzoic acid	300
α -Tocopherol	25
2-Methyl-1,4-naphthoquinone	2
Biotin	0.1
Folic acid ..	0.1

are not comparable to those procured when cystine was excluded from the food (1).

Three series of experiments, involving the use of more than 150 animals, were conducted. The results are summarized in Table III. In Series I the diets contained L-methionine in amounts varying from 0.1 to 1.4

per cent. Since eight levels were employed, it was not possible to distribute the members of each litter among the eight groups. However, such a distribution of litter mates was made, as equitably as possible, between rations containing contiguous percentages of methionine. All animals were from our own colony, which has been highly inbred for 25 years. Thus they are quite consistent in their growth behavior. Furthermore, the relatively large number of subjects receiving each diet, except the first, which obviously carried an inadequate amount of methionine, and the fact that males only were employed in the tests minimize the errors due to inherent differences in growth tendency.

TABLE III

Growth Effects of Diets Containing Different Levels of L- and D-Methionine
The experiments covered 28 days each.

Series No.	Isomer	Amount of isomer in diet	No. of animals (males throughout)	Mean gain in weight and probable error of mean	
		<i>per cent</i>			
I	L-Methionine	0.1	4	35.8	2.2
		0.2	16	98.4	1.3
		0.3	16	106.5	1.0
		0.4	16	109.0	1.8
		0.6	17	108.7	1.1
		0.8	19	104.7	1.8
		1.0	10	101.3	1.6
		1.4	10	92.7	2.0
II	D-Methionine	0.2	10	84.4	2.8
		0.6	10	105.2	2.3
		1.4	10	104.3	1.7
III	L-Methionine	0.2	10	91.9	3.0
	D-Methionine	0.2	10	88.1	2.5

An examination of the data (Series I) reveals a remarkable uniformity in the mean gains of the animals which received each level of L-methionine from 0.3 to 0.8 per cent inclusive. Indeed, the difference in the effect of 1.0 per cent, as compared to 0.4 per cent, is of doubtful statistical significance. On the other hand, 0.2 per cent is definitely inadequate under the conditions employed, and 1.4 per cent exerts an inhibitory action upon growth when contrasted with any percentage from 0.3 to 0.8 inclusive. With reference to the latter range, the moderate upward drift in the mean gains to a maximum, followed by a progressive downward drift as the proportion of L-methionine was further increased, appears to warrant the conclusion that dietary levels of 0.4 to 0.6 per cent of this amino acid are the most satisfactory. In establishing the *minimum* intake of any dietary

component, one should probably err in the direction of excessive liberality rather than risk the chance of adopting too low a figure. At best, values of this nature are approximations only, though extremely useful for many purposes. Because of these considerations, and despite the fact that a statistical analysis reveals little basis for a choice between 0.3 and 0.4 per cent, the latter should probably be regarded as the minimum level of L-methionine which is compatible with maximum growth.

In Series II, the effects of three levels of D-methionine were tested. The lower level, 0.2 per cent, was selected as an inadequate proportion, the intermediate level of 0.6 per cent as one which probably would be sufficient to support maximum growth, and the upper level, 1.4 per cent, as one which might exert a deleterious effect if its action were similar to that of L-methionine. The results of the tests are striking and clear cut. The lower and intermediate levels behaved as was anticipated, the former permitting subnormal gains only and the latter supporting weight increases which appear to be maximum. On the other hand, the upper level of 1.4 per cent, in contrast to the same proportion of L-methionine, exerted no retarding effect whatever upon growth. This confirms the findings obtained by Van Pilsum and Berg (8) and Wretling (9), under different experimental conditions, that a moderately excessive quantity of D-methionine is tolerated more readily by the rat than is a like amount of L-methionine. No explanation is apparent for this metabolic peculiarity of the two enantiomorphs

The growth obtained with 0.6 per cent of D-methionine seems to substantiate the conclusion reached in our earlier experiments (5); namely, that this optical isomer possesses a nutritive value which is essentially equal to that of L-methionine. However, it should be pointed out that the animals employed in Series II were not litter mates of those used in Series I. Indeed, the tests were conducted at different seasons of the year. Hence, the apparent agreement in the mean gains of the rats which received 0.6 per cent of the two stereoisomers (the divergence is not statistically significant) may have been fortuitous. That this is a possibility is suggested by the observation that the mean gains of the subjects (Series I and II) which received the compounds at the lower level of 0.2 per cent are not in close agreement, and appear to favor the group which received L-methionine. In order to clarify this situation, the tests summarized as Series III were undertaken. For this purpose, twenty males from several litters were divided into two groups which received diets containing 0.2 per cent of L- and D-methionine respectively. The mean gains in 28 days were 91.9 and 88.1 gm. The probable errors of the means were rather large, as frequently is the case when animals are placed upon partially deficient rations. Probably this is to be accounted for by the fact that some

subjects succeed in compensating in part for the dietary inadequacy by consuming larger amounts of the food. But despite this fact, the close agreement in the mean gains leaves little room for doubt that the two enantiomorphs are utilized by the species in question with essentially equal facility.

SUMMARY

Experiments have been conducted upon a relatively large number of male weanling rats for the purpose of determining the optimum dietary level of L-methionine for maximum growth, and the relative availability of its opposite enantiomorph. The diets uniformly contained 0.2 per cent of L-cystine.

The results demonstrate that a level of 0.2 per cent of L-methionine is definitely inadequate. On the other hand, no statistically significant differences were observed in the mean gains of the subjects with concentrations varying from 0.3 to 0.8 per cent. In view of the *tendency* toward slightly better growth with 0.4 than with 0.3 per cent, and in order to *insure* the presence in the food of a sufficient quantity of this amino acid, 0.4 per cent is proposed as the minimum level. The presence of 1.4 per cent exerts a distinctly inhibitory effect upon growth.

Under the conditions of our tests, D-methionine appears to be utilized for growth purposes by the species in question just as effectively as is L-methionine. In confirmation of the observations of others, obtained under different experimental conditions, a moderately excessive quantity of D-methionine is tolerated more readily by the rat than is a like amount of L-methionine.

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PHOTOMETRIC DETERMINATION OF CATALASE ACTIVITY

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Catalase activity may be measured quantitatively by the method of von Euler and Josephson (1) by allowing the enzyme solution to react with hydrogen peroxide for varying periods of time and measuring the excess peroxide remaining by titration with potassium permanganate. The similarity of this titration procedure to the assay of ascorbic acid by titration with 2:6-dichlorophenolindophenol suggested to the authors the possibility of measuring catalase activity photometrically (in a manner similar to the photometric estimation of vitamin C) by addition of an excess quantity of potassium permanganate and subsequent photometric measurement of the color. This procedure has been found to be practical and rapid. It gives reproducible results and, by photometric means, obviates the human error in reading the end-point of the potassium permanganate. As both hydrogen peroxide and potassium permanganate in the present procedure are used in the same concentrations as in the conventional von Euler and Josephson method, compounds that inhibit catalase such as HCN should have no different effect in this procedure from that in the conventional one.

Apparatus and Reagents—The photometric determination of catalase activity may be carried out with any standard colorimeter or spectrophotometer.¹

The reagents required for this measurement are as follows.

Hydrogen peroxide (approximately 0.01 N) in phosphate buffer at pH 6.8. Use 5.67 ml. of 3 per cent hydrogen peroxide per liter.

Potassium permanganate (approximately 0.005 N); 0.158 gm., made to 1 liter with distilled water.

A solution of diluted catalase enzyme. For our study, a 1:2500 catalase-Sarett² solution was used.

Sulfuric acid (5 N); 142 ml. of concentrated sulfuric acid (sp. gr. 1.84) made to 1 liter with distilled water.

¹ A Coleman spectrophotometer, a Cenco "photometer," or an Evelyn colorimeter will be found satisfactory for this purpose.

² Courtesy of the Vita-Zyme Laboratories, Inc., Chicago, Illinois.

Preparation of Standard Curve

In a volumetric flask, 10 ml. of hydrogen peroxide (3 per cent by volume) are diluted to 100 ml. with distilled water and mixed. Then into consecutive 100 ml. volumetric flasks are pipetted 6, 5, 4, 3, 2, and 1 ml., respectively, of the 0.3 per cent hydrogen peroxide solution. The contents of the flasks are made to volume with phosphate buffer (0.0067 M) and mixed. From each flask, a 5 ml. amount is pipetted into six reaction test-tubes containing 2 ml. of 5 N sulfuric acid. The photometer is set to transmit maximally at 515 μ and is adjusted to 100 per cent transmission with a

TABLE I
Typical Readings Obtained with Coleman Universal Spectrophotometer (Standard Curve)

Contents of tube, 17 ml.	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Blank	Reference tube
Ml. 0.3% H ₂ O ₂ per 100 ml..	6	5	4	3	2	1	0	
% H ₂ O ₂	0.018	0.015	0.012	0.009	0.006	0.003	0	
H ₂ O ₂ , ml.	5	5	5	5	5	5	0	0
Water, "	0	0	0	0	0	0	5	15
KMnO ₄ , "	10	10	10	10	10	10	10	0
H ₂ SO ₄ , "	2	2	2	2	2	2	2	2
Transmission, %	90°	83 ¹	75 ²	71°	66°	61°	57 ¹	100°
Optical density	0.0458	0.0796	0.1206	0.1478	0.1805	0.2147	0.2422	0.0000
D _{bl.} - D _{s.}	0.1964	0.1626	0.1216	0.0935	0.0617	0.0275		
Concentration	6	5	4	3	2	1		
Slope	0.0327	0.0325	0.0304	0.0312	0.0309	0.0275		
Average slope 0.0309								

reference tube which contains 2 ml. of 5 N sulfuric acid plus 15 ml. of distilled water.

Into one of the six reaction tubes, 10 ml. of the 0.005 N permanganate solution are blown. The contents are quickly mixed, and the transmission is read at once. This is repeated for all six tubes.

A seventh tube is prepared with 2 ml. of sulfuric acid, 5 ml. of water, and 10 ml. of permanganate. The transmission of this mixture is read and serves as the "blank."

Table I lists the contents of each of the tubes and presents a typical set of readings obtained with the use of a Coleman Universal spectrophotometer.

The optical densities which correspond to the transmissions, expressed in per cent, are calculated and can be plotted with reference to the rela-

tive concentrations of hydrogen peroxide in the test-tubes to obtain the "standard curve." As the relationship between optical density and percentage of concentration is linear in this case, it is actually unnecessary to plot the standard curve. All that is necessary is to calculate the amount of decrease in optical density below that of the blank for each increase of 1 per cent in relative concentration. This is done by subtraction of the value for the optical density of each concentration from that of the blank, and division of the result by the relative concentration. The resulting quotients or "slopes" are averaged to obtain the mean slope of the standard curve.

Measurement of Catalase Activity

The necessary dilutions of a preparation of catalase are made and placed in an ice-water bath. In this work, a 1:2500 solution of catalase-Sarett was used. To 50 ml. of 0.01 N hydrogen peroxide containing 0.007 M phosphate buffer at a pH of 6.8 (which has been allowed to sit in a beaker of ice water for 5 minutes) add 1 ml. of properly diluted enzyme. Mix, and immediately pipette 5 ml. of this solution into a reaction tube containing 2 ml. of the 5 N sulfuric acid. Mix, and add 10 ml. of the permanganate solution. Mix, and read in the colorimeter against the reference tube with a 515 m μ filter. Repeat again after 3, 6, 9, and 12 minutes. Then calculate the K_0 value and the *Kat. f.* value (2).

A blank reading is made with a mixture containing 2 ml. of acid, 5 ml. of distilled water, and 10 ml. of the permanganate solution.

The volume of permanganate remaining after the reaction with hydrogen peroxide is calculated according to the following formula:

$$\frac{D_{bl.} - D_s.}{\text{Slope}} = \text{ml. permanganate used}$$

where $D_{bl.}$ is the optical density of the blank reading, $D_s.$ is the optical density of the sample reading, and "slope" is the average slope of the standard curve calculated as described above.

Comparison of Measurements of Catalase Activity by Photometric Method and by Conventional Permanganate Titration Method

For comparative purposes the activity of a sample of "catalase-Sarett" was determined by the photometric method described above and by the conventional permanganate titration method. The results are summarized in Table II. As can be seen from the data, the *Kat. f.* values are in good agreement.

Precautions

Several precautions should be emphasized.

The time between pipetting of the permanganate solution into the tube and the photometric reading should be not more than 1 minute. After a period of from 1 to 1½ minutes, a colloidal suspension forms in the tubes, which is especially pronounced if the quantity of hydrogen peroxide remaining is significant.

TABLE II

Comparison of Measurements of Catalase Activity by Photometric and Permanganate Titration Methods

Reaction time	Photometric method				Titration method	
	Transmission	Optical density	KMnO ₄ used*	K _s .†	KMnO ₄ (0.005 N) used	K _s .†
min.	per cent		ml.		ml.	
0	86 ¹	0.064	5.95	0.0245	5.72	0.078
3	81 ⁰	0.092	5.06	0.02346	3.45	0.07319
6	77 ¹	0.112	4.39	0.02201	2.14	0.07083
9	74 ¹	0.129	3.84	0.02113	1.40	0.06792
12	70 ²	0.150	3.16	0.02040	1.00	0.06312
Blank	56 ²	0.248				
Kat. f.‡	$\frac{0.0245 \times 2500\%}{0.0271\%} = 2260$				$\frac{0.078 \times 1000\%}{0.0271\%} = 2880$	

$$* \frac{D_{bl.} - D_s.}{\text{Slope}}$$

† K_s. $\frac{1}{T} \log_{10} \frac{a}{a-x}$, where K_s. is the reaction rate, T time, a initial KMnO₄ concentration, and a - x is the KMnO₄ remaining. The K_s. value for T = 0 is obtained by extrapolation.

$$\ddagger \text{Kat. f.} = \frac{K_s. (0 \text{ time})}{\text{gm. enzyme (dry weight)}}. \text{ See Sumner and Somers (2).}$$

§ Enzyme dilution used was 1:2500 in the photometric method and 1:1000 in the titration method; the enzyme content was 0.0271 gm. (dry weight) per ml. in both instances.

One standard curve, if properly obtained with good duplicate checks and small deviations in slopes between the individual points, suffices for the particular instrument, wave-length, and permanganate pipette. A 10 ml., graduated pipette with a wide tip to facilitate rapid flowing is recommended.

Increasing or decreasing the permanganate concentration or increasing or decreasing the peroxide concentration has no effect on the slope of the curve, which is constant. Hence for such a determination, one has only

to ascertain the blank reading of permanganate, acid, and water and the reading of the unknown solution containing the reaction products of catalase activity.

SUMMARY

A technique is described for measuring catalase activity photometrically with the use of a Coleman spectrophotometer or any colorimeter.

A properly diluted enzyme solution is allowed to react with hydrogen peroxide for a specified period of time. The reaction is then stopped by use of a sulfuric acid solution. Potassium permanganate in excess is next added to the mixture and allowed to react with the peroxide not decomposed by the catalase. Within 1 minute after addition of the permanganate, the excess in potassium permanganate is determined photometrically.

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EXTRACTION AND PURIFICATION OF CORTICOTROPIN*

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The existence of a pituitary hormone which regulates the mammalian adrenal cortex was first demonstrated by Smith (1) in 1927; the implantation of pituitary glands restored to normal the atrophic adrenal cortices of hypophysectomized rats. Enlargement of the adrenals following the injection of pituitary extracts was observed by Houssay *et al.* (2) in the hypophysectomized dog and by Collip, Anderson, and Thomson (3) in the hypophysectomized rat. Collip *et al.* found the corticotropic hormone to be unusually stable to heat and to be difficult to extract and to purify. Anselmino, Hoffmann, and Herold (4) also concluded that the hormone is stable to heat and concluded that it, unlike other anterior pituitary hormones, would pass an ultrafilter. This conclusion, though based upon assays with non-hypophysectomized animals, was substantiated by Tyslowitz (5) and by Cortis-Jones, Crooke, Henly, Morris, and Morris (6), using improved methods of corticotropin assay.

Of the various extraction procedures which have been applied to obtain solutions of the active principle the more successful have involved vigorous treatment of the fresh or acetone-dried pituitary tissue. Thus, Fevold, Lee, Hisaw, and Cohn (7), employing the classical methods of protein chemistry, obtained low yields of corticotropin from sheep pituitaries, and Chow, Greep, and van Dyke (8), using a saline extract and salting-out procedures followed by dialysis, could barely detect corticotropin in their extracts of pig pituitaries. Collip (9) obtained more active materials by extraction with 80 per cent ethanol or 70 per cent acetone at pH 2.5, while Bates, Riddle, and Miller (10) employed alkaline aqueous ethanol. Lyons' successful procedure (11), involving 83 per cent acetone and strong hydrochloric acid, was subsequently used by Li, Evans, and Simpson (12) and by Sayers, White, and Long (13) to obtain a pure protein which was considered to represent the true hormone.

None of the published procedures has permitted the recovery of corticotropin in good yield, and no methods have been described for the prep-

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aration of highly active materials. Earlier work was seriously hampered by difficulties in assay; the elegant method devised by Sayers, Sayers, and Woodbury in 1948 (14), while difficult to carry out, has provided for the first time a reliable and sensitive test for the active principle.

The impressive therapeutic effects of corticotropin have emphasized the desirability of developing efficient and simple methods for the extraction of the hormone in a form suitable for clinical use, and have generated increased interest in the preparation of purified materials for chemical, physiological, and clinical studies.

Earlier observations (15, 16) had indicated that the pituitary of the pig contained more corticotropin than that of any other readily available animal, and that corticotropic activity declined in pituitary glands preserved by freezing. It had further been observed, in confirmation of the findings of Collip, that corticotropin was removed from the acetone-dried powder with great difficulty by simple aqueous solvents unless strongly acid or alkaline solutions were employed, but that glacial acetic acid efficiently dissolved the active principle.¹

In view of these considerations acetone-dried pig pituitary powder² was extracted at 70° with glacial acetic acid. From this solution impurities were precipitated by acetone after the addition of a small amount of salt, and the active fraction then was obtained by precipitation with ether. This preparation, referred to below as crude corticotropin, contained nearly all of the activity of the starting material and was more active than the pure protein preparations referred to above; it was freely soluble in water and was sufficiently free of undesirable contaminants to be suitable for clinical use (17). An extensive therapeutic investigation was carried out in patients with this preparation without significant side effects attributable to impurities.

Fractionation of the crude extract by precipitations from aqueous solution failed to give clean separations of impurities without substantial losses. Isoelectric precipitation removed a variable fraction of the total activity from solution. Sodium chloride in concentrations below 1 M removed inert material from 0.1 N hydrochloric acid solutions without great loss, but higher salt concentrations failed to precipitate all of the active principle. Precipitation from acid aqueous solutions by the addition of alcohols or acetone was likewise unsuccessful. Dialysis in acid solutions with cellophane did not lead to an appreciable concentration of the active principle in the dialysate, though some highly colored non-dialyzable impurities could thus be removed.

¹ Tislow, R., and Astwood, E. B., unpublished results.

² Generously supplied by Dr. David Klein of The Wilson Laboratories, Chicago, Illinois.

Fractionation on starch columns by the techniques of Stein and Moore (18) did not lead to good resolution. Under most conditions a large fraction of the total extract together with some active principle traversed the columns unadsorbed, while under other conditions it was not possible to recover all of the activity. Similar behavior was observed when powdered cellulose was used with organic solvent mixtures. It was noted, however, that cellulose would remove the active principle quantitatively from dilute acetic acid solutions of the extract; subsequent elution with dilute hydrochloric acid yielded the active principle in a form representing a 10-fold increase in purity. This simple and reproducible procedure provided a means of preparing large quantities of concentrate for further fractionation.

Purification of this hydrochloric acid eluate could be achieved by taking advantage of the altered solubility properties of the active fraction in the presence of organic acids. Picric acid precipitated the material from aqueous solution and this acid as well as benzenesulfonic, sulfosalicylic, and trichloroacetic acids greatly increased solubility in alcohols. Such fractionations followed by counter-current distributions with the Craig principle (19) made it possible to achieve a further 4-fold increase in potency, but without convincing evidence of homogeneity.

Attempts to identify the active principle on paper chromatograms were unsuccessful. The recognizable ninhydrin-reactive spots obtained from the hydrochloric acid eluate, as well as from crude corticotropin, were produced by contaminating materials.

The activity of crude corticotropin and of the hydrochloric acid eluate was completely destroyed by prolonged digestion with crystalline pepsin in 0.1 *N* hydrochloric acid. Brief digestion produced a marked decrease in the amount of material precipitable by trichloroacetic acid, sulfosalicylic acid, or by adjustment to pH 6.0 to 6.5, before a decrease in corticotropic activity was detectable.

Bioassays by the method of Sayers, Sayers, and Woodbury (14), though subject to considerable error, indicated that the acetone-dried anterior pituitary powder used as starting material was about 40 per cent as active as the Armour standard Preparation La-1-A and the pure proteins referred to above. The 5-fold concentration provided by the glacial acetic acid method for the preparation of the crude extract and the 10-fold increase in potency given by cellulose adsorption followed by the further 4-fold purification through the use of the organic acids represent a total purification of 200 times. Thus, three steps serve to yield, without major losses, extracts about 80 times as active as the Armour Preparations La-1-A.

It appears unlikely that the pure protein described by Li, Simpson, and Evans and by Sayers, White, and Long could have been the true corticotropic hormone in view of the preparation of materials of considerably

greater potency. The suggestion by Li (20) that more active substances represent fragments of the protein hormone of molecular weight of 20,000 seems improbable, since an 80-fold increase in potency has been achieved in the course of this investigation without encountering evidence that the material obtained is in the range of a molecular weight of 250. Similarly, this material must have contained a more active molecule than the pure polypeptide described by Morris and Morris (21), which they found to be 8.5 times as active as Preparation La-1-A.

EXPERIMENTAL

Chemical Analyses—Three procedures, in addition to dry weight, were used to measure the material contained in the various fractions: (1) the biuret reaction was carried out by the method of Gornall, Bardawill, and David (22) with crystalline pepsin as a standard; (2) the ninhydrin method was that of Moore and Stein (23) with glycine as a standard; and (3) absorption at a wave-length of $275\text{ m}\mu$ was measured in acid aqueous solution with the Beckman spectrophotometer, model DU. These procedures were employed with the full realization that the active principle might not be detected by one or all of them. However, the purest fractions still contained material which reacted with ninhydrin and the biuret reagent and showed an absorption maximum at $275\text{ m}\mu$.

Assay Methods—The assays for corticotropic activity were made by the method of Sayers, Sayers, and Woodbury (14) with minor modifications. The hypophysectomized test animals were used 1 day after the operation; they were anesthetized with ether, and a wide transverse incision was made over the right adrenal. The adrenal was removed quickly, adhering tissue removed and the adrenal weighed; it was then placed in a 12 cc., thick-walled centrifuge tube containing 6 cc. of 4 per cent trichloroacetic acid and a small amount of clean sea sand. The animal was supported by grasping the vertebral column between the thumb and forefinger, allowing the wound to gape open, which provided easy access to the inferior vena cava. The test substance, diluted in 0.9 per cent sodium chloride solution containing 1:5000 acetic acid in a volume varying from 0.1 to 1 cc., was injected into the vena cava and the skin incision closed with four skin clips. 1 hour after the injection the left adrenal was removed under chloroform anesthesia and treated as the first. Each adrenal was ground with an individual, new, birch applicator stick. The ground preparation was centrifuged and the supernatant was shaken with 150 mg. of activated charcoal and filtered. A 4 cc. aliquot was taken for ascorbic acid determination by the method of Roe and Kuether (24).

It was desirable, in using this method, to compare unknown fractions with a standard, preferably at several dose levels. Such comparisons were

made with a few preparations, but it was not practical or possible to carry out such extensive assays on the numerous fractions encountered during the course of this investigation. Instead, rough estimates of activity were made by determining the minimal effective dose. Dilutions were made so that decimal fractions of 1, 2.5, and 5 γ quantities were given; responses representing a fall of less than 60 mg. per cent of adrenal ascorbic acid were considered to be negative; when the ascorbic acid depletion exceeded 100 mg. per cent, the tests were repeated with smaller doses; the test was considered satisfactory when the value for the ascorbic acid depletion was within the range of 60 to 100 mg. per cent.

Thyrotropin was determined in day-old chicks by the method of Bergman and Turner (25). Prolactin was assayed in pigeons by the subcutaneous method of Bates and Riddle (26). Gonadotropin determinations were performed on hypophysectomized, 21 day-old, male rats as described by Greep, van Dyke, and Chow (27).

Preparation of Crude Extract—In a representative preparation, 250 cc. of acetone were added to 100 gm. of acetone-dried anterior pituitary powder to make a paste; 1600 cc. of glacial acetic acid were added slowly and mixed vigorously and the mixture was heated to 70° on the steam bath with continuous stirring and with precautions to prevent entry of water vapor. The mixture was filtered through Hyflo Super-Cel after cooling to about 35°, and the cake was washed with 200 cc. of glacial acetic acid and then with 200 cc. of acetone. To the combined filtrates (1950 cc.) were added 10 cc. of 5 M aqueous NaCl and 1000 cc. of acetone. When the solution had stood overnight in a refrigerator, the precipitate, which contained thyrotropin, follicle-stimulating hormone, luteinizing hormone, and prolactin, but little corticotropin, was removed by filtration and to the clear filtrate was added an equal volume of ether. The precipitate settled quickly and after 2 hours the supernatant was siphoned; the precipitate was collected on a sintered glass funnel and thoroughly washed with acetone to remove acetic acid. The precipitate could not be allowed to dry or become exposed to the air until the acetone washing was complete; it then was quickly transferred to a desiccator and dried over NaOH with the vacuum of a water pump. The dry powder was twice as active as Preparation La-1-A. It was virtually free of other anterior pituitary hormones except for traces of prolactin and was sufficiently depleted of pressor and oxytocic hormones to be satisfactory for clinical use.

With different amounts of starting material, the quantities of reagents were changed proportionally. Small variations in yield were encountered (Table I), but the activity of various batches was so uniform that significant differences in potency could not be detected by the bioassay used or by the responses of patients being treated for various disorders.

In Table I are listed the quantities of material precipitated by acetone and the yields of the crude corticotropin obtained in thirteen experiments. The values were similar with batches varying from 20 to 300 gm. of pituitary powder. Larger quantities of sodium chloride increased the weight of the acetone precipitate and the corticotropic fraction contained salt. Smaller quantities of salt reduced the quantity of solids precipitated by acetone. Somewhat greater volumes of acetone were used in some batches.

TABLE I

Preparations of Crude Corticotropin from Anterior Lobe Pituitary Powder

Precipitation from glacial acetic acid extracts of inactive material with acetone and the active fraction with ether, expressed in percentage by weight of starting material.

Preparation No.	Starting material	Quantity pptd.	
		By acetone	By ether
	gm.		
11-19*	25	9.2	12
12-15*	25	7.6	13.9
1-11	20	7.5	13.0
2-1†	25	8.8	11.3
2-16	200	6.8	12.95
1-18	100	6.6	14
1-28‡	100	3.8	15.4
3-20	100		11.5
3-7	200	7.0	13.0
2-6	150	9.0	11.0
5-24	300		13.6
6-9	300		13.9
1-3*‡	100	8.0	14.3

* Double the usual quantity of NaCl used; extracts contained salt.

† One-half the usual quantity of NaCl used; acetone precipitation incomplete.

‡ From whole pituitary powder.

Purification by Adsorption on Cellulose—Preliminary attempts to employ cellulose for chromatographic separations indicated that some active material was firmly adsorbed by this substance and not easily eluted. Investigation of this phenomenon showed that a constant quantity of the total solids of the crude extract was retained by a given weight of cellulose and that the active material was quantitatively retained if sufficient cellulose were used. Thus, when 40 mg. quantities of the extract in 0.1 N acetic acid were applied to columns containing 1, 10, and 20 gm. of cellulose, the quantities retained by the columns were 1.8, 17.7, and 34.2 mg. respectively. The quantities retained by 1 gm. of cellulose were thus 1.8,

1.77, and 1.71 mg. It was further observed that when the quantity of cellulose was in excess of 50 times the weight of the extract virtually all of the active material was retained in the column. The finding that elution of the active fraction could be accomplished by dilute hydrochloric acid permitted the use of cellulose adsorption as an efficient method of purification, as shown in Fig. 1.

In an illustrative experiment (Preparation 4-1, Table II) a chromatographic column 7.5 cm. in diameter and 25 cm. high was prepared with 300 gm. of purified powdered cellulose⁸ which had been serially washed on a large, sintered glass funnel with 3 liters each of 0.1 N HCl, H₂O, and 0.1 N

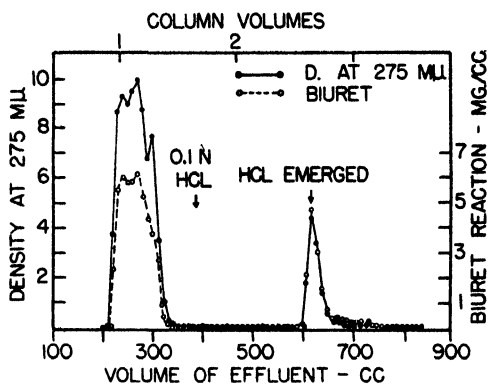


FIG. 1. Purification of crude corticotropin by adsorption on powdered cellulose from 0.1 N acetic acid and elution with 0.1 N hydrochloric acid. Column 3.3 cm. in diameter and 32 cm. high containing 75 gm. of cellulose; column volume 235 cc. Crude corticotropin, 1.44 gm., applied in 24 cc. of 0.1 N acetic acid, followed by 390 cc. of 0.1 N acetic acid and then 300 cc. of 0.1 N hydrochloric acid. The inactive acetic acid fraction totaled 0.953 gm. and the active hydrochloric acid eluate 0.125 gm. Preparation 1-25, Table II.

acetic acid. The column was poured as a thick slurry in 0.1 N acetic acid and packed with a partial vacuum.

5.7 gm. of crude extract were dissolved in 100 cc. of 0.1 N acetic acid and after application to the column were washed through with 2 liters of 0.1 N acetic acid. 4.1 gms. of material (as estimated by the biuret reaction) emerged with the acetic acid and was virtually free of corticotropic activity. 200 cc. of water were then run through, followed by 0.1 N HCl. When the water began to appear, as indicated by test paper, the eluate was collected in small fractions until all of the water and about 200 cc. of the HCl had emerged. The fractions containing significant concentrations

⁸ Solka-Floc BW-200, obtained from the Brown Company, Berlin, New Hampshire.

of material as measured by the biuret reaction were combined to yield 300 cc. of a solution containing 524 mg. of material of activity 10 times that of the crude extract. In Table II are shown the yields obtained in eleven experiments. It may be noted that from 5.1 to 9.7 per cent of the extract emerged with the 0.1 N hydrochloric acid and that, with the exceptions noted in Table II, the yield was similar whether 1.5 or 25 gm. were thus treated. As the activity was increased by about 10-fold by this procedure, the recovery was between 50 to 100 per cent of the total of the crude extract. This material could be obtained in the dry state by

TABLE II

Adsorption of Crude Corticotropin on Powdered cellulose from 0.1 N Acetic Acid and Elution of Active Fraction by 0.1 N Hydrochloric Acid

Preparation No.	Dimensions of column	Weight of cellulose	Weight of crude extract	Amount recovered		Minimal effective dose
				In acetic acid	In HCl eluate	
	cm.	gm.	gm	per cent	per cent	γ
1-25	3.3 $\times$ 32	75	1.4	67	8.7	0.1
2-9	7.5 $\times$ 19	225	4.0	82	8.5	0.025
2-19	7.5 $\times$ 21	250	5.0	82	9.0	0.05
2-24	7.5 $\times$ 21	250	5.0	76	7.9	0.05
2-25	3.3 $\times$ 22	50	1.0	75	9.7*	0.05
3-14	13.2 $\times$ 7	250	5.5	79	7.6	0.1
3-15	3.3 $\times$ 22	50	1.1†	69	5.1*	0.1
3-29	3.4 $\times$ 86	200	5.0		5.9	0.025
4-1	7.5 $\times$ 25	300	5.7	72	9.2	0.1‡
5-24	14.2 $\times$ 30	1250	25.0	71	7.7	0.05‡
6-10	8.4 $\times$ 80	1250	25.0		8.0	0.05

* 0.1 N H₂SO₄ used instead of HCl.

† Crude extract first treated in 0.1 N acetic acid with 1/80 its weight of pepsin for 30 minutes.

‡ Greater than minimal response; lower doses not tested.

first removing most of the hydrochloric acid with an anion exchange resin (Amberlite IRA-400) and then dried while frozen.

Preparation of Picrate—100 cc. of the 0.1 N HCl eluate (180 mg.) were treated with 5 mg. of pepsin at 37° for 30 minutes, reduced to 30 cc. in a vacuum, and NaOH added to pH 6.5 (brom cresyl purple indicator paper); after the small precipitate was removed, 300 mg. of picric acid were added with thorough mixing. The precipitate was removed and dissolved by stirring with 5 cc. volumes of mutually saturated 2-butanol and water. The aqueous phase was discarded and the 2-butanol phase was carried through eighteen transfers according to the Craig counter-current distribution principle (19) with 5 cc. portions of mutually saturated 2-butanol and

0.05 per cent aqueous picric acid. The distributions as determined by the ninhydrin reaction were plotted as in Fig. 2. Assays showed that the activity had concentrated in the vicinity of Tube 15. A positive test was given by 0.01 γ , indicating a potency above 80 times that of preparation La-1-A. Upon evaporating the 2-butanol phase of Tube 15 and removal of most of the free picric acid with ether, a solid remained which was sparingly soluble in hot water and more soluble in aqueous alcohols. After separation from water by cooling to remove traces of free picric acid and drying, this material had lost activity. The weight corresponded to that estimated by the ninhydrin reaction and the material contained

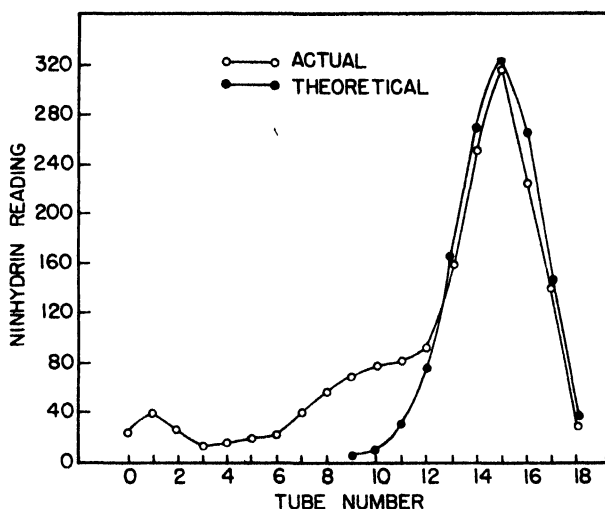


Fig. 2. An eighteen transfer distribution of material purified by cellulose adsorption and elution, followed by picric acid fractionation. System, 5 cc. volumes of mutually saturated 0.05 per cent aqueous picric acid and 2-butanol. The major component contained the active principle. A theoretical curve calculated by the method of Williamson and Craig (28) is shown for comparison.

33 per cent picric acid as determined spectrophotometrically. No method was found for obtaining the picrate in dry form without loss of potency.

Conversion of Picrate to Acetate—To the 2-butanol solution of the picrate was added an equal volume of water, and the anion exchange resin, Amberlite IRA-400, in the form of the acetate was then added in small amounts with shaking until the 2-butanol phase was free of color. The 2-butanol was discarded and the aqueous phase, together with water washings of the resin, was lyophilized. The faintly buff-colored powder had retained the corticotropic activity without detectable loss. This powder was

freely soluble in water, giving a clear and nearly colorless solution of about pH 5.

Purification by Means of Benzenesulfonic Acid—The contents of three tubes of an eight transfer picric acid distribution were combined, and the picrate converted to the acetate as described in the preceding section. The material was then distributed between 0.03 molar aqueous benzene-sulfonic acid and 2-butanol, with a 20 per cent excess of the acid in the starting tube. After twelve transfers, there appeared to be a partial separation between two substances, with estimated distribution ratios of aqueous to

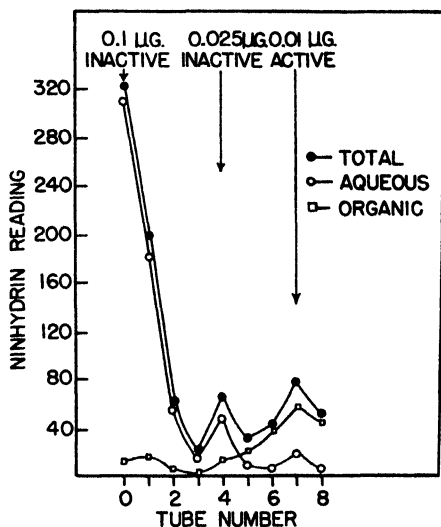


FIG. 3. An eight transfer counter-current distribution of hydrochloric acid eluate after removal of material precipitated by 0.05 M sulfosalicylic acid. Solvent system, equal volumes of 0.05 M aqueous sulfosalicylic acid and 2-butanol. It is to be noted that the material was separated into two major inactive fractions and a small highly active component.

organic phase of 25:1 and 6:1 respectively. Assay indicated that the activity was in the less hydrophilic fraction, and Tube 0 (the starting tube) was therefore dropped, and the distribution extended for eighteen more transfers. After the thirty transfers, the major component of the remaining material exhibited a distribution curve, with a ninhydrin peak in Tube 5 and a fairly constant aqueous-organic distribution ratio of 6.5 in Tubes 4 to 8.

Purification with Sulfosalicylic Acid—30 cc. of the hydrochloric acid eluate containing 45 mg. were concentrated by evaporation in a vacuum to 5 cc. The solution was adjusted to pH 4 with 3 per cent NaOH. Sulfosalicylic acid, when added to yield a 0.05 M solution of the acid, pro-

duced a precipitate which was removed by centrifugation after chilling in an ice water bath and discarded. The precipitate contained 35 mg. of the material according to the biuret reaction. (In subsequent preparations, precipitation was made from more dilute solutions of the extract and smaller fractions were removed.) An eight transfer distribution of the supernatant, with 5 cc. of each phase of mutually saturated 0.05 M aqueous sulfosalicylic acid and 2-butanol, separated the activity from the major portion of the material (Fig. 3). The active fraction gave a positive assay

TABLE III
*Corticotropic Assays of Various Preparations by Method of Sayers, Sayers,
and Woodbury*

	No. of animals	Dose	Mean depletion of adrenal ascorbic acid, per 100 gm. fresh tissue $\pm$ s.e.	Estimated relative potency*
		γ	mg.	
Anterior lobe powder	8	2.5	78.8 $\pm$ 15.3	1
Armour, Preparation 60-61	6	1	67.3 $\pm$ 15.7	1.5
	7	0.5	10.25 $\pm$ 32.8	
	3	0.25	8.3 $\pm$ 20.6	
-1-A	10	1	54.9 $\pm$ 7.5	2.5
	8	0.5	60 $\pm$ 12.95	
	3	0.25	26 $\pm$ 6.1	
Crude extract	19	0.5	80.3 $\pm$ 8.52	5
	11	0.25	56.4 $\pm$ 12.81	
Hydrochloric acid eluate	6	0.1	120.1 $\pm$ 23.4	50
	20	0.05	80.6 $\pm$ 6.77	
	8	0.025	64.3 $\pm$ 14.85	
Purified picrate	5	0.025	98.6 $\pm$ 12.9	150
	11	0.01	60.4 $\pm$ 6.79	
Purified sulfosalicylate	7	0.025	106.3 $\pm$ 20.2	200
	7	0.01	71 $\pm$ 9.9	

* Compared to acetone-dried pig anterior pituitary powder.

at a dose of 0.01 γ ; it was more stable than the picrate and retained activity when evaporated to dryness.

Comparative Assays—While assays on well over a thousand animals were successfully completed, the limited number of animals which could practicably be employed for any one preparation decreased precision and the values obtained are regarded only as semiquantitative.

A representative comparative assay of the various preparations described, as well as of pig anterior pituitary powder, and of two Armour preparations,⁴ Nos. La-1-A and 60-61, is presented in Table III. Table

⁴ Preparations 60-61 and La-1-A were supplied through the kindness of Dr. Edwin Hays of Armour and Company. Dr. Hays had found Preparation 60-61 to be equal in

III contains the results of a group of assays, and the estimated relative potencies are expressed in relation to the potency of anterior pituitary powder assigned a value of 1. It may be noted that anomalous results were obtained in two cases; thus, 1.0 γ of Preparation La-1-A produced a smaller response than half that dose, and 0.5 γ of Preparation 60-61 failed to give a significantly greater response than 0.25 γ . Accurate comparisons of the various preparations with these two standards are therefore difficult. Satisfactory dose-response relationships were obtained for the other materials tested.

We are indebted to Dr. David Klein of The Wilson Laboratories for abundant supplies of pituitary powders and for his helpful advice, to Dr. R. O. Roblin and his associates of the Stamford Research Laboratories, American Cyanamid Company for generous advice and assistance, and to Dr. R. Tislow for permission to cite earlier unpublished observations.

SUMMARY

Extraction of acetone-dried pig anterior pituitary powder, with a corticotropic potency 0.4 times the Armour standard| Preparation La-1-A with glacial acetic acid at 70° followed by precipitation of impurities with 0.5 volume of acetone and the active fraction with 1 volume of ether, gave a water-soluble powder representing 11 to 15 per cent of the weight of original powder, with a 5-fold increase in potency. Adsorption on powdered cellulose from 0.1 N acetic acid and elution with 0.1 N hydrochloric acid increased the activity 10-fold with little loss. Further fractionation by means of solvent partition in the presence of picric, benzenesulfonic, and sulfosalicylic acids led to the isolation of concentrates 150 to 200 times as active as the starting material.

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PROLINE AND HYDROXYPROLINE: DETERMINATION OF THE SUM OF THEIR α -NITROGEN

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A method for the quantitative determination of the α -imino carboxyl nitrogen¹ of free proline plus hydroxyproline in the presence of other amino acids is presented. This method, reported in abstract (2), is an extension of the studies on proline and hydroxyproline already published (3). Employing similar preparative techniques, Chinard² has developed a colorimetric ninhydrin method for the determination of proline.

Primary amino groups of α -amino acids react with nitrous acid to yield nitrogen gas and usually the corresponding α -hydroxy derivatives, whereas proline and hydroxyproline, depending upon the acid strength, are either unchanged, or, like secondary amines, form relatively unstable *N*-nitroso derivatives. In this method, elimination of α -amino groups takes place in boiling 6 N HCl, excess nitrous acid is decomposed by ammonium sulfamate, and the minimal amounts of *N*-nitroso proline and *N*-nitroso hydroxyproline formed are hydrolyzed by further heating in 6 N HCl. Unidentified products of the reaction, which interfere in the subsequent analysis, are extracted with ether and the proline and hydroxyproline remaining are determined quantitatively by the ninhydrin method (3, 4).

Recovery of proline or hydroxyproline is 97.3 and 96.3 per cent, respectively, of the theoretical. The loss is apparently due to destruction of small portions of the imino acids. Correction factors of 1.028 and 1.038, respectively, are valid when checking the purity of these acids. In mixtures of amino acids, *e.g.* protein hydrolysates, small positive errors measured as imino nitrogen are contributed by threonine, tyrosine, tryptophan, histidine, lysine, methionine, cysteine, cystine, arginine, citrulline, and ornithine, but the sum of these positive errors in most cases very nearly compensates for the loss of imino nitrogen. For mixtures of known composition, it is preferable to subtract the computed sum of positive errors of the above α -amino acids from determined imino nitrogen and to increase

¹ In conformity with nomenclature of previous publications (Hamilton (1), footnote p. 375) the term "imino carboxyl nitrogen" is used to indicate values calculated as 1 gm. atom of nitrogen per molecule of CO₂ evolved by reaction of the α -imino acids, proline and hydroxyproline, with ninhydrin.

² Personal communication from F. P. Chinard, School of Medicine, Johns Hopkins University.

the resulting value by 3 per cent to correct for the average loss of proline and hydroxyproline nitrogen.

For optimal gas pressures in the manometric ninhydrin method (4), approximately 2 mg. of proline or hydroxyproline are necessary and this amount is contained in 50 to 60 mg. of most proteins; because of its high imino acid content, 10 mg. of gelatin suffice.

Hillier and Van Slyke (5) determined by Kjeldahl analysis the non-amino nitrogen (*i.e.*, imino nitrogen) remaining in filtrates after precipitation of the basic amino acid fraction of acid hydrolysates of proteins. As stated by them, determination was limited to solutions which contained only primary amino groups capable of being eliminated by nitrous acid; the presence of secondary nitrogen atoms of arginine, histidine, or tryptophan, for example, would give erroneously high values.

Apparatus—The Van Slyke-Neill (6) manometric apparatus, together with apparatus for ninhydrin determinations (4), including all-glass reaction vessels and a high temperature lubricant (7), is necessary. A micro-Kjeldahl digestion rack (Arthur H. Thomas, No. 7498) for gas heating and 100 ml. micro-Kjeldahl flasks are also needed.

Reagents—Solid citrate buffer, pH 2.5, octyl alcohol, 5 N NaOH (7), 0.5 N NaOH of minimal CO₂ content and nearly saturated with sodium chloride (8), and 2 M lactic acid, also nearly saturated with sodium chloride and containing hydrazine (9), are needed.

Ninhydrin.³ Recrystallized repeatedly from 2 N HCl until 100 mg. yield no CO₂ when tested in a blank analysis (10).

Sodium nitrite solution, 3 M. 20.7 gm. of sodium nitrite (NaNO₂, analytical grade) dissolved in water and made to 100 ml. of solution.

Hydrochloric acid solutions. Concentrated hydrochloric acid and solutions of approximately 8.6 N, 6 N, 3 N, and 1 N.

Ammonium sulfamate solution, 60 per cent. 60 gm. of ammonium sulfamate (NH₂SO₂NH₄, LaMotte, pure standardized) dissolved in water and made to 100 ml. of solution.

Diethyl ether. Distilled before use.

Brom cresol green indicator, 0.04 per cent, aqueous.

Procedure

Preparation of Substances for Analysis—50 to 60 mg. of protein are refluxed in a 100 ml. Kjeldahl flask with 5 ml. of 6 N HCl for 24 hours. Otherwise, 5 ml. portions of hydrochloric acid hydrolysate or solutions of substances in 6 N HCl are placed in 100 ml. Kjeldahl flasks.

Conversion of α -Amino Acids to α -Hydroxy Acids—1.5 ml. of 3 M sodium

³ Ninhydrin from Dougherty Chemicals, 87-134th Street, Richmond Hill 18, New York, has been satisfactory without further purification.

nitrite solution and 3.5 ml. of 8.6 N HCl are mixed in a small beaker, and, after the vigorous reaction subsides, the mixture is added to the flask. The solution is boiled over a micro burner for exactly 3 minutes; white alundum grains are added to prevent bumping. Longer heating favors the production of larger amounts of interfering substances from arginine, citrulline, or ornithine.

Elimination of Excess Free Nitrous Acid—0.5 ml. of 60 per cent ammonium sulfamate is added, and the flask immersed for 30 minutes in boiling water.

Hydrolysis of N-Nitroso Derivatives—30 to 40 ml. of 6 N HCl are added and the flask boiled vigorously. 6 N HCl is added as necessary but fluid is allowed to boil away until 3 ml. remain after 90 minutes. The minimal amounts of *N*-nitroso proline or hydroxyproline formed during reaction with nitrous acid are hydrolyzed.

Extraction of Interfering Substances by Ether—The remaining 3 ml. of acid are removed *in vacuo* directly at a water pump, suction being discontinued when the residue is still somewhat moist in appearance. 1 ml. of 3 N HCl is introduced and manipulated to wet all the adherent residue, followed by 50 to 60 ml. of ether which is swirled vigorously for 1 minute and then discarded. Extraction is repeated twice more.

Preparation of Solutions for Final Analysis—Residual ether and acid are removed at the pump, and the residue is dissolved in 5 ml. of water, again reduced to dryness, and finally redissolved in 2 ml. of water. 2 drops of brom cresol green indicator are added and the color adjusted to green by the addition of 5 N NaOH and 1 N HCl. The solution is transferred to a volumetric flask and made to 11 ml. with water.

Determination of Carboxyl Nitrogen of Proline Plus Hydroxyproline—A 5 ml. portion of solution is placed in each of two all-glass ninhydrin reaction vessels. Reaction with ninhydrin (100 mg.) is carried out at pH 2.5 for exactly 6 minutes at 100° (3) and the CO₂ evolved is measured manometrically as previously described (Hamilton and Van Slyke (7) pp. 238–239), and recorded as $p_1 - p_2$.

*Blank Determination and Measurement of *c* Correction*—The *c* correction is the $p_1 - p_2$ value, obtained by analysis of blank solutions of 6 N HCl, carried through the entire procedure.

Calculations—The pressure P_{CO_2} of CO₂ from the imino acid carboxyl groups in the sample analyzed is calculated as $P_{\text{CO}_2} = p_1 - p_2 - c$. The mg. of carboxyl nitrogen = $P_{\text{CO}_2} \times \text{factor}$. The values for the factors are given in Column 2, Table I, of the paper by Hamilton (1). Imino carboxyl nitrogen in the 11 ml. of solution, equivalent to 5 ml. of the original hydrolysate, is therefore $P_{\text{CO}_2} \times \text{factor} \times 2.20$. For hydrolysates of proteins of established composition, the mg. of nitrogen contributed by threonine,

tyrosine, tryptophan, methionine, cystine, cysteine, histidine, lysine, arginine, ornithine, and citrulline are calculated by means of the values given in Column 2, Table II, and their sum, α , is computed. If N represents the total nitrogen in the 5 ml. of hydrolysate analyzed and the recovery of imino carboxyl nitrogen is 97 per cent, then the imino carboxyl nitrogen as per cent of total nitrogen is given by

$$[P_{\text{Co}_2} \times \text{factor} \times 2.20 - \alpha] \frac{103}{N}$$

TABLE I
Recovery of Proline and Hydroxyproline Imino Carboxyl Nitrogen

Determination No.	Proline nitrogen	Hydroxyproline nitrogen
	Determined N Theoretical N $\times 100$	Determined N Theoretical N $\times 100$
	<i>per cent</i>	<i>per cent</i>
1	97.6	96.8
2	97.9	96.7
3	97.2	95.7
4	97.4	96.2
5	97.5	95.7
6	96.3	96.1
7	97.4	96.9
8	96.3	96.3
9	97.6	95.8
10	97.4	96.6
Average*	97.3	96.3

* The average values shown form the basis for calculating correction factors as described in the text.

Simplified Procedure As Test of Purity of Proline or Hydroxyproline—5 ml. of 6 N HCl solution containing approximately 2 mg. of proline or hydroxyproline are treated as described with the exception that the 30 minute heating at 100° and the ether extraction are omitted. Imino carboxyl nitrogen is determined as above. Proline values are increased by 2.8 per cent, hydroxyproline values by 3.8 per cent, to correct for losses in carboxyl nitrogen.

EXPERIMENTAL

Recovery of Proline and Hydroxyproline—The ten determinations (each the average of duplicate ninhydrin analyses) in Table I show that recovery of proline or hydroxyproline imino carboxyl nitrogen is 97.3 and 96.3 per cent of theoretical, respectively. Hence, correction factors of 1.028 and

1.038 are valid if the purity of proline or hydroxyproline is being checked. A mean factor of 1.03 is sufficiently exact when imino carboxyl nitrogen is

TABLE II

Recovery of Proline and Hydroxyproline Imino Carboxyl Nitrogen Added to Amino Acids

Amino acid (1)	Imino N of amino acids, $\frac{\text{Imino N}}{\alpha\text{-N}} \times 100$ (2)	Recovery* of 0.2 mg. imino N added to 2.64 mg. α -nitrogen	
		Proline	Hydroxyproline
		$\frac{\text{N found}^*}{\text{N calculated}} \times 100$ (3)	$\frac{\text{N found}^*}{\text{N calculated}} \times 100$ (4)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Glycine	0.00	100.4	99.2
DL-Alanine	0.00	100.8	99.9
DL-Leucine	0.00	100.3	100.0
DL-Isoleucine	0.00	102.0	98.2
DL-Norleucine	0.00	100.3	98.7
DL-Valine	0.00	101.2	101.0
DL-Serine ..	0.00	100.3	98.1
L-Glutamic acid	0.00	100.6	100.6
L-Glutamine	0.00	97.9	97.3
DL-Aspartic acid	0.00	101.3	102.3
DL-Asparagine	0.00	100.5	99.7
DL-Phenylalanine	0.00	100.3	100.4
DL-Threonine ..	0.18	99.6	99.3
DL-Tryptophan	0.93	100.6	97.7
L-Tyrosine ..	0.80	99.7	102.1
DL-Methionine ..	0.23	99.8	98.2
L-Cystine ..	0.30	99.8	98.2
L-Cysteine ..	0.14	100.4	98.4
L-Histidine ..	0.37	100.5	98.9
L-Lysine ..	0.22	97.0	98.0
L-Arginine ..	0.90	100.8	99.9
DL-Ornithine	2.07	100.0	100.4
DL-Citrulline	2.35	100.4	96.7
Average ..		100.2	99.3

* All recoveries, Columns 3 and 4, are corrected for plus errors contributed by α -amino acids (Column 2) and loss of proline or hydroxyproline.

determined in mixtures of amino acids. This loss of proline or hydroxyproline is greater than that reported previously (3) owing apparently to

the greater destructive effect of nitrous acid in hot 6 N HCl compared with the weakly acid (pH 2.5) condition necessary for quantitative formation of the *N*-nitroso derivatives.

Recovery of Proline and Hydroxyproline Added to Single Amino Acids, Synthetic Mixtures, and Protein Hydrolysates—Tables II and III show that recovery of proline or hydroxyproline, added to single amino acids,

TABLE III

Recovery of Proline and Hydroxyproline Imino Carboxyl Nitrogen Added to Amino Acid Mixtures and Acid Protein Hydrolysates

Amino acid solution	Imino N added		Recovery of imino N added	
	Proline	Hydroxyproline	Uncorrected, $\frac{\text{found N}}{\text{calculated N}} \times 100$	Corrected for imino N of α -acids and imino N loss, $\frac{\text{found N}}{\text{calculated N}} \times 100$
(1)	(2)	(3)	(4)	(5)
	mg.	mg.	per cent	per cent
Synthetic mixture*	0.251		99.6	102.0
	0.251		99.1	101.5
	0.232		101.6	103.9
	0.050		100.2	98.2
		0.251	97.2	99.2
		0.251	98.9	100.4
		0.216	98.2	100.0
		0.380	95.0	97.9
		0.050	105.2	103.4
Gelatin hydrolysate	0.1157		98.7	102.2
Silk hydrolysate	0.1184		96.2	99.2
		0.1281	97.0	100.0
Bovine plasma albumin	0.1122		98.1	101.1
		0.1281	97.8	100.8
Edestin hydrolysate	0.1266		97.3	100.3
		0.1063	95.8	98.7
Average			98.5	100.5

* Contains the first eighteen amino acids of Table I.

synthetic mixtures, or acid protein hydrolysates, is usually within ± 2 per cent of theoretical. Positive errors introduced by some of the amino acids, calculated as per cent of their α -nitrogen, are shown in Column 2, Table II. It is unlikely that the imino nitrogen determined is due to contaminating proline or hydroxyproline, for, in every case, hydrolysis for

TABLE IV
Imino Carbozyl Nitrogen of Animal Proteins

Protein	Source*	Total nitrogen, corrected for moisture and ash	Imino N Total N $\times 100$		Values from literature
			Un-corrected	Corrected†	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Albumin, egg	E. A.	14.94	2.93	2.94	2.78 (15)
	N. B.	14.58	3.11	3.13	
“ blood	E. A.	14.49	3.98	4.01	
“ bovine plasma	Armour No. 46	15.89	3.82	3.86 $\pm$ 0.05	3.60 (16), 4.24 (17), 3.86 (18), 4.16 (19)
	“ “ G4802	15.73	3.82	3.83 $\pm$ 0.05	
	“ “ G4502	15.84	3.93	3.96	
Lactalbumin	N. B.	12.25	4.38	4.42	
β -Lactoglobulin	Stein-Moore (16)	15.60	4.08	4.14	4.01 (16), 3.20 (20), 4.24 (21), 3.78 (22)
Fibrin, blood	C. C.	14.92	3.74	3.76	4.11 (for
	N. B.	16.32	3.89	3.94	fibrino- gen) (15)
Hemoglobin	Merck	16.23	2.97	3.01	2.83 (15)
Keratin	“	11.98	2.63	2.64	
Casein, Hammarsten	E. A.	15.46	8.39	8.57	9.7 (23)
Peptone	Witte	16.18	4.54		
Silk	N. B.	18.36	0.51	0.22	0.48 (15)
Protamine SO ₄	N. B.	23.77	2.99	2.88	2.30 (for salmine) (15)
Gelatin	Knox	17.61	17.8	18.3 $\pm$ 0.01	18.7 (23)
	N. B.	18.43	17.0	17.5 $\pm$ 0.03	
	E. K. (foam)	17.58	18.9	19.3 $\pm$ 0.06	
	“ (pig skin)	18.47	18.0	18.5 $\pm$ 0.08	

* E. A., Eimer and Amend; N. B., Nutritional Biochemicals Corporation; C. C., City Chemical Corporation; E. K., Eastman Kodak Company.

† Values corrected for plus errors contributed by α -amino acids and imino nitrogen loss. Corrections for α -amino acids of egg albumin, bovine plasma albumin, hemoglobin, silk, and gelatin calculated from their composition as given by Tristram (15). It was considered sufficiently exact to assume blood albumin similar to bovine plasma albumin, fibrin similar to fibrinogen, protamine-SO₄ similar to salmine, and keratin similar to hair as given in the literature (15). Composition of lactalbumin, edestin, and gelatin obtained from Chibnall (23), β -lactoglobulin from Stein and Moore (16).

24 hours in 6 N HCl, followed by isolation and recrystallization of the amino acid, did not significantly alter the value. Arginine was further purified through the flavianic acid and benzylidene derivatives (11), ornithine as the picrate (12), and citrulline as the copper salt (13), all without change. Unknown side reactions are presumably responsible for these findings. The interference of arginine, citrulline, and ornithine is being investigated further.

Imino Carboxyl Nitrogen Values of Proteins—Tables IV and V show corrected and uncorrected imino nitrogen values obtained for hydrochloric

TABLE V
Imino Carboxyl Nitrogen of Vegetable Proteins

Protein	Source	Total nitrogen, corrected for moisture and ash	$\frac{\text{Imino N}}{\text{Total N}} \times 100$		Values from literature]
			Un- corrected	Corrected*	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Gluten, wheat	City Chemical Corporation	14.31	9.70	9.94	10 (24)
	Nutritional Biochemicals	15.43	9.13	9.35	
Zein	Eastman Kodak	15.54	7.94	8.14	7.90 (15)
	Nutritional Biochemicals	16.90	7.92	8.12	
Globulin, pumpkin seed	Difco	18.31	3.59		
Edestin	Nutritional Biochemicals	18.73	2.92	2.90	2.79 (15)

* Values corrected for plus errors contributed by α -amino acids and imino nitrogen loss. The corrections for α -amino acids of zein and edestin were calculated from their composition as given by Tristram (15); wheat gluten from its composition as given by Block and Bolling (24).

acid hydrolysates of some proteins of animal and vegetable origin. Total nitrogen of the proteins was determined by the macro-Kjeldahl method (14) and corrected for moisture and ash content. The β -lactoglobulin analyzed is a portion of the same preparations previously investigated in great detail (16, 20); the 15.6 per cent nitrogen reported for this protein was accepted without further verification.

The figures for gelatin and bovine plasma albumin (Table IV) are the averages obtained on analysis of two or three different hydrolysates of the same proteins. For these two proteins, the maximal and minimal deviations from the average are indicated.

Imino Carboxyl Nitrogen of Tissues—In Table VI, values for some tissues

are given. Preparatory to hydrolysis, cortical bone (steer and human) was stripped of periosteum, marrow, trabeculae, and endosteum. Beef articular disk and tendon were trimmed of fat and loose fibrous tissue. Hair and wool were washed and ether-extracted for 24 hours in a Soxhlet apparatus. Reconstituted human, pooled, lyophilized plasma was dialyzed in a cellophane sac for 3 days at 4° against repeated changes of distilled water; the dialysate was concentrated and then hydrolyzed.

TABLE VI
Imino Carboxyl Nitrogen of Tissues

Tissue	Imino N Total N $\times 100$		Values from literature, Imino N Total N $\times 100$
	Uncorrected	Corrected*	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Tibia, beef cortical bone (Argentine)	19.4	19.9	
" " " " (local)	16.4	16.8	
Humerus, human cortical bone	16.4	16.8	
Articular disk, beef	16.0	16.4	
Tendon, beef	16.8	17.2	
Skin, beef	16.6	16.8	
" pig	16.1	16.5	
Hair, human	3.10	3.09	
" sheep wool	5.30	5.34	7.09 (15)
Plasma, human, lyophilized	4.74	4.79	
" " dialysate	0.63†		

* Values corrected for plus errors due to α -amino acids and imino nitrogen loss. Corrections for α -amino acids of bone proteins, articular disk, tendon, and skin were assumed to be similar to that calculated for gelatin (15), human whole plasma similar to fibrinogen (15), sheep wool as in (15) and human hair as in (23).

† Mg. of N per 100 ml. of plasma (uncorrected).

SUMMARY

1. A method for the determination of total α -imino carboxyl nitrogen (proline nitrogen plus hydroxyproline nitrogen) in mixtures of amino acids, *e.g.* hydrochloric acid protein hydrolysates, without preliminary isolation or separation, is presented. Under the conditions of analysis, nitrous acid destroys the ability of α -amino acids to react with ninhydrin, whereas proline and hydroxyproline are essentially unchanged and their α -nitrogen is quantitatively measured by the manometric ninhydrin-CO₂ method (4).

2. The 2 mg. of proline or hydroxyproline needed for a determination are, in most cases, contained in 50 to 60 mg. of protein or in 10 mg. of gelatin and closely related proteins. Results within ± 2 per cent can be readily obtained.

3. α -Imino carboxyl nitrogen of proteins determined by this method agrees well with values reported in the literature obtained by other methods.

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LIVER ARGINASE ACTIVITY AS RELATED TO BLOOD UREA IN ACUTE UREMIA OF NEW-BORN RATS*

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There is evidence to indicate that the demands imposed upon the mammalian organism to metabolize protein may be reflected by changes in the arginase activity of the liver. Particularly those conditions leading to a stimulation of protein catabolism, with its concomitant increase in the formation of urea, frequently elicit a corresponding increase in the arginase activity of the liver. Thus, elevations in liver arginase activity have been observed under the dietary influence of fasting (1, 2), increased levels of protein intake (3, 4), and thiamine deficiency (5). Hormonal secretions, known to control the rate of gluconeogenesis, effect a rise in the arginase content of the liver when injected into hypophysectomized or adrenalectomized animals (6-9).

Previous reports from this laboratory have described a high incidence of early mortality of rats born to mothers maintained on rations composed mainly of crude or purified plant materials (10-12). A characteristic feature of the syndrome which preceded death, referred to as "acute uremia of the new-born," was the very high level of urea in the blood. The subcutaneous administration of vitamin B₁₂ shortly after birth was effective in preventing the onset of the uremic syndrome. If this uremic condition actually reflects an increased rate of nitrogen catabolism which can be prevented by vitamin B₁₂, it appeared possible that, under such circumstances, the arginase activity of the liver might be increased in order to accommodate the need for this enzyme.

In the present paper data are reported on the relationship between arginase activity of the liver and the concentration of urea in the blood of normal and uremic young rats.

EXPERIMENTAL

Animals and Rations—The care and feeding of the animals, as well as the rations used in these experiments, have been described elsewhere in detail (12). The basal ration designated Ration CS-1 was composed of the following ingredients, gm. per kilo of diet: ground yellow corn, 753.4;

* Paper No. 2576, Scientific Journal Series, Minnesota Agricultural Experiment Station. This is the sixth of a series of papers dealing with the nutritional value of plant materials.

expeller soy bean meal, 175.0; alfalfa meal, 50.0; salt mixture (12), 21.6. In Ration CS-3, the protein content was increased by raising the level of the soy bean meal to 375.0 and lowering the corn to 553.4 gm. per kilo of diet. Vitamins A and D were supplied as 2 drops of halibut liver oil (Abbott) per rat per week. Previous experience has shown that in about 40 per cent of the litters born to females maintained on these rations from one to all of the young incur symptoms of acute, fatal uremia (12). The addition to these basal rations of 3 per cent condensed fish solubles or the early postnatal administration of 0.05 γ of crystalline vitamin B₁₂ could prevent this uremic syndrome.

Arginase Activity—As soon as the external symptoms of the acute uremia became apparent, usually within 24 to 48 hours following birth, the animals were decapitated and the blood was collected for the determination of urea in the manner described below. The livers were quickly excised and weighed to the nearest mg. The livers weighed from 100 to 250 mg. The whole liver was homogenized in 5 ml. of ice-cold redistilled water. In order to activate the enzyme an equal volume of 0.1 M $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ was added and the mixture incubated for 1 hour in a water bath, equipped with a shaking device at 38°.¹ A 1 ml. aliquot of the activated homogenate was diluted to 50 ml. with 0.85 per cent NaCl solution. A 1 ml. aliquot of this diluted homogenate was used for measuring the arginase activity, according to the colorimetric procedure described by Van Slyke and Archibald (13). The arginase activity was expressed in terms of the number of arginase units, as defined by Van Slyke and Archibald (13), per mg. of fresh tissue. Calculation of arginase activity on the basis of the weight of dried tissue gave essentially the same results.

Normal appearing young from females maintained on the basal ration supplemented with 3 per cent fish solubles were sacrificed within 24 to 48 hours after birth and the arginase activity of the livers determined in the manner described.

Blood Urea—Following decapitation, the blood was collected into small oxalated glass vials (16 mm. outside diameter and 1 cm. deep). A volume of the blood measured to the nearest 0.001 ml. (volumes of samples ranging from 0.050 to 0.150 ml.) was removed from the vial in an 0.2 ml. Kahn serological pipette and immediately laked in 2 ml. of distilled water. The subsequent steps involving dilution, deproteinization, and the determination of urea have already been described in detail (14).

¹ Several comparative experiments were conducted on liver homogenates which had not been activated in this manner. Although the arginase activity in such instances was about half that of the activated homogenates, the difference in arginase activity between normal and uremic animals was of the same magnitude as in the activated samples.

Results

Effect of Fish Solubles on Liver Arginase Activity and Blood Urea—The data presented in Table I summarize the influence of the maternal diet on liver arginase activity and the concentration of blood urea of young rats within 24 to 48 hours after birth. The uremic new-born from mothers raised on the unsupplemented rations containing the crude plant materials displayed the marked elevation of urea in the blood which is characteristic of this syndrome. Mothers on similar rations supplemented with 3 per cent fish solubles in no instance produced young with acute uremia, as evidenced either by their external appearance or by the concentration of urea in the blood, as shown in Table I. The marked elevation in blood urea in the uremic animals was accompanied by a significant increase in the arginase activity of the liver.

TABLE I
Liver Arginase Activity and Blood Urea Concentration of Young Rats Born to Mothers Fed Rations Containing Crude Plant Materials with and without Fish Solubles

Ration	No. of animals	Condition of animals	Liver arginase activity*	Blood urea*
			units per gm. fresh tissue	mg. per 100 ml.
CS-1, unsupplemented.	30	Uremic	840 $\pm$ 126	225.8 $\pm$ 49.2
“ + 3% fish solubles	30	Normal	472 $\pm$ 76	70.8 $\pm$ 38.8
CS-3, unsupplemented	19	Uremic	870 $\pm$ 165	234.0 $\pm$ 66.4
“ + 3% fish solubles	8	Normal	448 $\pm$ 37	75.2 $\pm$ 10.7

* Mean $\pm$ standard deviation.

Variation of Arginase Activity Following Birth—Since it had been previously reported that there is a transient rise in the concentration of urea in the blood of new-born rats during the first few days after birth (12), it appeared likely that this transitory increase in urea concentration might also be reflected by the arginase activity of the liver. A study of this relationship, as influenced by the presence or absence of fish solubles in the maternal diet, was made by analyzing the blood and liver of young from the same litter killed at various intervals. A graphic presentation of the data, obtained by these analyses extending over a 48 hour period following birth, is shown in Fig. 1. The changes in concentration of blood urea of normal and uremic rats followed a course similar to that reported previously (12). Closely paralleling these changes in concentration of blood urea were those which occurred in the activity of arginase in the liver. An increase in arginase activity was observed within 12 to 24 hours after

birth, coinciding with the marked rise in the blood urea which occurred during the same period. This increase in arginase activity was particularly marked in those rats observed in a state of acute uremia, but appreciably less in normal appearing young from others on the same unsupplemented ration. The presence of fish solubles in the maternal diet permitted a more gradual rise in arginase activity in the livers of the new-born. Whereas the concentration of urea in the blood of normal animals tends to return to a lower level within 24 to 48 hours following birth, there appeared to be no immediate tendency for liver arginase activity to decline

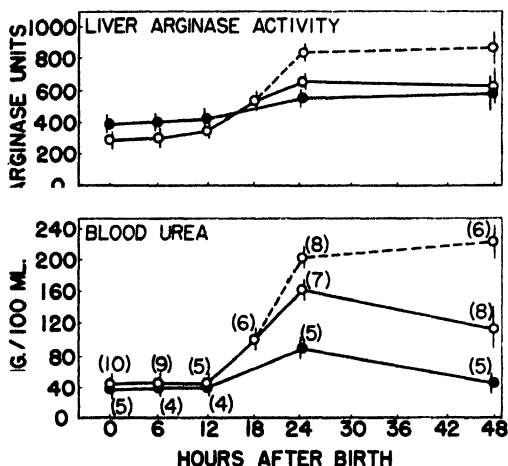


FIG. 1. Changes in concentration of blood urea and liver arginase activity of the new-born rat. Basal Ration CS-1 was used in this experiment. The figures in parentheses indicate the number of animals used in calculating the mean value represented by each point. The vertical lines through each point indicate the magnitude of the standard deviation. O, solid line, young of normal appearance on basal ration; O, dash line, young with acute uremia on basal ration; ●, young of normal appearance on basal ration + fish solubles.

once maximum activity had been attained. According to the data of Lightbody (15), who also observed a rise in liver arginase activity in rats during the first few days of life, one might expect a gradual return to the level found at birth by the 9th to the 18th day.

Effect of Crystalline Vitamin B₁₂ on Liver Arginase Activity and Blood Urea—In this experiment, 0.1 ml. of a solution containing 0.06 γ of crystalline vitamin B₁₂ (Cobione, Merck) was injected subcutaneously within 2 to 3 hours after birth into about half the number of young in each litter from mothers on Ration CS-1. The other half of the litter served as a control. In the 48 hour period following birth, animals observed in the acute uremic

crisis were sacrificed at once. All animals appearing normal were sacrificed at the end of 48 hours. As shown by the data in Table II, vitamin B₁₂, like condensed fish solubles, prevents a continued rise of arginase

TABLE II
Effect of Vitamin B₁₂ on Liver Arginase Activity and Blood Urea of Young Rats Born to Mothers Fed Ration CS-1

Observed condition	Injected with 0.06 γ of vitamin B ₁₂			Uninjected controls		
	No. of animals	Liver arginase activity*	Blood urea*	No. of animals	Liver arginase activity*	Blood urea*
		units per gm. fresh tissue	mg. per 100 ml.		units per gm. fresh tissue	mg. per 100 ml.
Total of live young born	30			34		
Young with acute uremia.	0			23	731 $\pm$ 153	211.4 $\pm$ 28.4
Normal young at end of 48 hrs.	28	502 $\pm$ 196	123.2 $\pm$ 41.8	7	510 $\pm$ 122	128.0 $\pm$ 26.2

* Mean $\pm$ standard deviation.

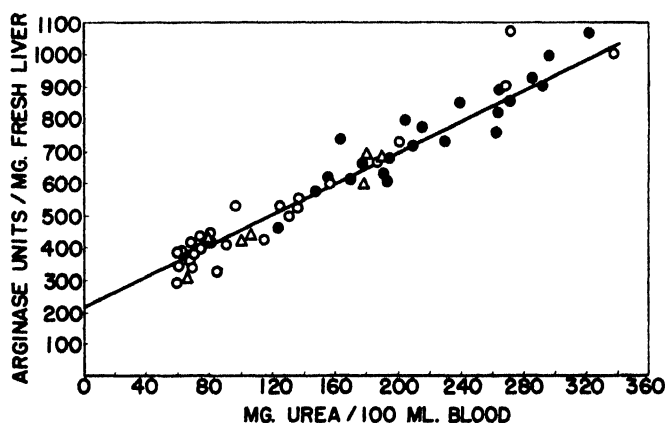


FIG. 2. The effect of crystalline vitamin B₁₂ on concentration of blood urea and liver arginase activity of the new-born rat. Correlation coefficient (r) = +0.86 (r at 1 per cent level of significance = +0.35). The equation expressing the regression of arginase units per gm. of fresh liver (y) on the concentration of blood urea as mg. per 100 ml. of blood (x) is $y = 208 + 2.44x$. ●, uninjected young with acute uremia; Δ, uninjected young with normal appearance; ○, vitamin B₁₂-injected young with normal appearance.

activity, as well as the appearance of the external symptoms of acute uremia.

The close relationship that exists between arginase activity in the liver

and the concentration of urea in the blood is evident from Fig. 2, a scatter diagram of individual determinations which constitute the basis for the average values shown in Table II. A noteworthy feature revealed by Fig. 2 is that, in spite of the absence of the external features of acute uremia, several of the animals receiving vitamin B₁₂ had concentrations of blood urea equivalent to those usually found in uremic young. The same was true for some of the normal appearing young which had not been injected with vitamin B₁₂. In the case of young born to mothers on basal rations supplemented with fish solubles, however, blood urea levels equivalent to those found in uremic young were never encountered. It would appear that the quantity of vitamin B₁₂ injected, although sufficient to prevent the onset of the uremic crisis as manifested by external appearances, is not sufficient in all instances to prevent the accumulation of large amounts of urea in the blood. On this basis, therefore, one can account for the fact that the mean values for blood urea and liver arginase activity in new-born rats injected with vitamin B₁₂ (Table II) are somewhat greater than those obtained from animals produced by females on the same basal diet supplemented with fish solubles (Table I).

DISCUSSION

Elevated concentrations of non-protein nitrogenous constituents have been observed in the blood of rats (16) and chicks (17, 18) maintained on rations devoid of animal protein. Hartman, Dryden, and Cary (19) and Bosshardt, Paul, and Barnes (20) found that increasing the protein level of diets deficient in vitamin B₁₂ accentuated the requirements for vitamin B₁₂. Charkey *et al.* (18) have interpreted these results, as well as their own, to indicate that vitamin B₁₂ may function in metabolism by enhancing the utilization of circulating amino acids for the synthesis of tissue protein.

The present data show that the accumulation of large quantities of urea in the blood, and the increase in arginase activity which accompanies it, occur only after the young have nursed. Since it has been consistently observed in this laboratory that, on rations producing acute uremia of the young, the early lactation of the females is excellent (11, 12), it appears that the elevation in blood urea and liver arginase activity is associated with the sudden influx of nutrients from the alimentary tract. In animals deficient in vitamin B₁₂, the failure to utilize absorbed amino acids² for

² This contention is supported by preliminary observations which indicate that the blood of uremic new-born rats had concentrations of non-urea non-protein nitrogen and α -amino nitrogen, determined by the method of Frame *et al.* (21), which were about double those found in the blood of normal appearing young of the same age.

protein anabolism might be expected to bring into play those mechanisms which facilitate their elimination from the body in the form of urea. The close relationship demonstrated to exist between liver arginase activity and the concentration of urea in the blood is strong indication that one of the adjustments that the organism makes in order to cope with this defect in nitrogen metabolism is to increase the arginase activity in its liver.

SUMMARY

The acute uremia observed in new-born rats from mothers maintained on rations containing crude plant materials was accompanied by a significant increase in the activity of liver arginase, compared to rats of normal appearance of the same age in which the maternal diet had been supplemented with fish solubles.

Liver arginase activity closely paralleled the marked rise in the concentration of urea in the blood which occurred 12 to 24 hours after birth.

The early postnatal administration of vitamin B₁₂ prevented the abnormal rise in arginase activity and prevented acute uremia in the new-born rat. Under these conditions a high degree of correlation was found to exist between the arginase activity of the liver and the concentration of urea in the blood.

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UTILIZATION OF HOMOCYSTINE FOR GROWTH IN PRESENCE OF VITAMIN B₁₂ AND FOLIC ACID*

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It has been shown in this laboratory that rats bred on our preexperimental food will grow on a synthetic diet in which homocystine is the only sulfur-containing amino acid and which contains no known sources of "labile methyl" groups (1, 2). The ability to utilize homocystine is eventually lost on the synthetic diet, probably due to the exhaustion of stored factors involved in homocystine metabolism. However, this ability to utilize homocystine can be restored by supplementation of the diet with liver extract Lilly (LEL). The liver preparation represents the antianemia fraction formerly called Cohn fraction G (2). In order to find out whether the antianemia principle in the LEL is the active agent in the utilization of homocystine, two Lederle preparations were also fed to rats on a homocystine-synthetic diet containing 2 per cent sulfasuxidine. One preparation, liver extract parenteral (Lederle), is similar to LEL, the other, Lederle concentrated solution of liver extract, is a product derived from the preceding preparation low in folic acid but 4 to 5 times as high in antianemia potency. Although the liver extract parenteral gave a growth response similar to that of LEL when given both orally and by intramuscular injection, the concentrated solution of liver extract gave negative results with both methods of administration (2). It was assumed from these results that the component of the liver preparations active in the utilization of homocystine was not identical with the antianemia principle, although its distribution parallels that of the latter to some degree.

Since this original work was published, crystalline folic acid and vitamin B₁₂ have become available. When the previously cited experiments were carried out, 2 per cent sulfasuxidine was added to the basal diet to inhibit possible bacterial synthesis of factors involved in the utilization of homocystine. At that time, ryzamin-B was our source of folic acid and the intestinal synthesis of additional amounts was inhibited by the sulfasuxidine. Recently some observations were made on the rôle of crystalline

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folic acid in the utilization of homocystine by the rat (3) in the presence and absence of sulfasuxidine, with and without the addition of LEL. It was found that doses of 20 γ of crystalline folic acid enabled some rats, which had lost their ability to utilize homocystine for growth, to reestablish this capacity without the aid of LEL. The fact that only *some* rats could resume growth seems to indicate that other factors were involved. "These animals retain a latent capacity for the utilization of homocystine which seems to depend for its manifestation on a fairly high level of folic acid and is not destroyed by the sulfa drug." The involvement of other B vitamins, particularly vitamin B₁₂, has been suggested (3) and, therefore, crystalline vitamin B₁₂, cobione (Merck), was added to the synthetic diet.

The present experiments were run to determine the combined effect of crystalline vitamin B₁₂ and folic acid on the ability of the rat to utilize homocystine on a "labile methyl"-free diet. In view of our last experiments with folic acid (3), the addition of 2 per cent sulfasuxidine was considered unnecessary. However, for the sake of comparison with former work experiments were carried out in both the presence and the absence of sulfasuxidine. The experiment with the concentrated solution of liver extract (Lederle), which yielded negative results in early experiments (2), was repeated in the presence of crystalline folic acid.

Diet

The composition of the basal (17 per cent) amino acid diet was the same as that used in previous experiments and from the same sources (2-4). DL-Homocystine was fed in the concentration of 0.83 per cent and was prepared in this laboratory (100 ± 0.5 per cent by disulfide determination (3)). When sulfasuxidine was fed, it was added to the basal diet which was given *ad libitum*.

The standard daily dose of B vitamins fed was 500 γ each of nicotinic acid, *p*-aminobenzoic acid, and inositol, 200 γ of calcium pantothenate, 40 γ each of thiamine hydrochloride, riboflavin, and pyridoxine hydrochloride, and 2 γ of biotin. 62.5 mg. of unfortified ryzamin-B (Wellcome Research Laboratories) and 20 γ of folic acid (folvite, Lederle) were later added to the vitamin supplement; in one group they were added at the beginning of the experiment. The preparation of vitamin B₁₂ used was cobione (Merck); the liver extract was Lederle concentrated solution liver extract (2).

EXPERIMENTAL

Three groups of female rats born and bred in this laboratory were used, four animals in each group. The rats were 35 days old at the beginning of the experiment. The preexperimental conditions were the same as

those previously described (2-4). The rats were allowed to lose weight for 14 days on the basal diet, which contained no sulfur-containing amino acids. In Groups A and B, 2 per cent sulfasuxidine was added to the basal diet. After 14 days 0.83 per cent homocystine was also added to the diet and the amount of food given was restricted to 3 gm. per rat per day. Rats receiving sulfasuxidine grew slowly for approximately 2 weeks, when the usual drop in weight occurred. At this point, 62.5 mg. of ryzamin-B and 20 γ of folic acid per day were administered to each rat for 3 to 4 days in order to aid recovery. The rats were then kept on the

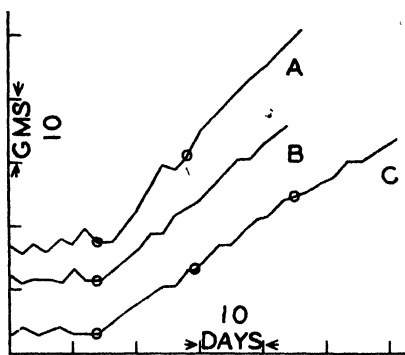


FIG. 1. Average daily growth curves. The basal diet contained 0.83 per cent homocystine and, in Groups A and B, 2 per cent sulfasuxidine. All supplements were given orally. The circles represent a change of period. The initial period in all the curves represents a leveling of the growth in the presence of daily doses of 62.5 mg. of ryzamin-B, 2 γ of biotin, and 20 γ of folic acid added to the vitamin B group supplement. Curve A, second period, represents the growth response of rats to a daily dose of 0.1 cc. (2 γ) of the Lederle concentrated solution of liver extract per rat and the third period, that to 0.2 cc. (4 γ) of the same solution. Curve B, second period, represents the growth response to 2 γ of vitamin B₁₂ (cobione) per day per rat. Curve C, second period, shows growth response to 2 γ of vitamin B₁₂ (cobione) per day per rat, which was increased in the third period to 4 γ of vitamin B₁₂, when it was discontinued (fourth period).

original supplement of the vitamin B group and given the basal food *ad libitum*. When the growth curve had leveled (in approximately 50 days), it was assumed that the sulfasuxidine had become effective and, therefore, at this point, ryzamin-B and additional biotin were added, followed in a short time by folic acid (2-4). If the growth curve of the rats remained level upon the addition of these extra vitamins, the animals were considered ready for vitamin B₁₂ assay. Those rats which did not receive the sulfa drug were fed the full supplement of B vitamins, including ryzamin-B, folic acid, and biotin, from the beginning of the experimental period. After the rats had been kept for 14 days on the basal diet free

of sulfur-containing amino acids, 0.83 per cent homocystine was added. The growth curves of these rats are usually level in about 40 to 50 days. When the homocystine was first fed, the basal food was limited to 5 gm. per rat per day; after approximately 20 days the animals were fed *ad libitum*. The food consumption averaged about 6.0 gm. per day for each rat during this period.

The four rats represented by Curve A, Fig. 1, were conditioned on the 2 per cent sulfasuxidine diet (initial period) and then given individual daily oral doses of 0.1 cc. of Lederle concentrated solution of liver extract containing 2 γ of vitamin B₁₂ according to biological assay (second period). This amount, which was added to the vitamin supplement, was increased to 0.2 cc. (4 γ of vitamin B₁₂) (third period). The rats weighed 120, 130, 110, and 132 gm. when conditioned and showed a daily gain of 0.8, 1.0, 0.9, and 0.8 gm. when supplemented with 0.1 cc. of Lederle solution. A daily gain of 1.5, 1.1, 0.9, and 0.8 gm. was recorded with 0.2 cc. of Lederle solution. The average gain for 2 γ of vitamin B₁₂ (0.1 cc.) was 0.9 and that for 4 γ of vitamin B₁₂ (0.2 cc.) 1.1 gm.

Four rats were used in Group B. Curve B, Fig. 1, represents two of these rats. The animals weighed 122 and 120 gm. after having been conditioned on the 2 per cent sulfasuxidine diet (initial period). They were given daily doses of 2 γ of vitamin B₁₂ for 30 additional days (second period); the gain per day averaged 0.86 and 0.80 gm. The other two rats fed a shorter period showed a similar growth response to 2 γ of vitamin B₁₂.

The four rats, the growth of which is represented by Curve C, Fig. 1, were conditioned as described without sulfasuxidine (initial period). When they had leveled, 2 γ of vitamin B₁₂ were added to the daily vitamin supplement of each rat for a period of 15 days (second period), after which the amount was increased to 4 γ of vitamin B₁₂ for 16 additional days (third period). The initial weight at the beginning of the vitamin B₁₂ assay was 158, 158, 124, and 131 gm. respectively; the average gain per day with 2 γ of vitamin B₁₂ was 0.7, 0.8, 0.4, and 0.5 gm. When 4 γ of vitamin B₁₂ were administered, the average daily gain was 0.8, 0.8, 0.6, and 1.1 gm. The average gain for the two periods was 0.6 and 0.8 gm. respectively.

DISCUSSION

Rats fed a "labile methyl"-free diet containing 0.83 per cent homocystine as the sole source of sulfur amino acids and the eight B vitamins gradually stopped growing if their intestinal synthesis of folic acid was suppressed by sulfasuxidine and none was given in the diet. When folic acid was added to the diet, some of the rats resumed growth while others did not (3). However, all the animals eventually ceased growing. This

seemed to indicate that an additional factor or factors were involved. Addition of crystalline vitamin B₁₂ to the vitamin supplement, at this point, caused a resumption of growth in all the animals at the rate of approximately 0.8 to 1.0 gm. daily (Curves B and C, Fig. 1). Removal of vitamin B₁₂ resulted in a continued growth for a short period, followed by a slow leveling of the curves. If an equimolar amount of methionine was fed with the basal diet instead of the homocystine at the point mentioned above, there was a resumption of growth of approximately 1 gm. a day without the addition of vitamin B₁₂.

Since vitamin B₁₂ has been shown to be the antianemia principle in liver preparations (5), it seems probable that the component of our liver extracts active in the utilization of homocystine was vitamin B₁₂. It has been shown that liver extracts rich in the pernicious anemia factor are only partially effective in anemia when given alone, but in combination with folic acid give complete recovery (6). In our former experiments, when the Lederle concentrated solution liver extract was used, as stated before, our source of folic acid was obviously inadequate. In the present experiment (Curve A, Fig. 1), growth was obtained with the Lederle liver extract proportional to its vitamin B₁₂ content, on addition of folic acid. Our data seem to indicate that, as in the case of pernicious anemia, the growth response of rats to vitamin B₁₂ on the homocystine diet seems to depend on the folic acid supply whether the source is from intestinal synthesis or from the diet. Our earlier negative experiments with the Lederle extract can now be explained in the light of our present experience with folic acid.

Since our diet was devoid of methionine and all known "labile methyl" donors, including choline, it appears that the growth response obtained in our animals is indicative of the synthesis of methionine in the rat. Since slow growth was obtained, it is obvious that this synthesis is limited but nevertheless real. Further studies on this problem are at present in progress.

SUMMARY

When growth had leveled on a "labile methyl"-free diet containing folic acid, addition of crystalline vitamin B₁₂ promoted growth.

The Lederle concentrated solution of liver extract given orally in place of vitamin B₁₂, under similar conditions, also promoted growth which was apparently proportional to its vitamin B₁₂ content.

It appears that the previous negative results obtained with the Lederle concentrated solution of liver extract (2) were due to folic acid deficiency.

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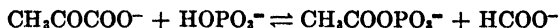
COENZYME A DEPENDENCE AND ACETYL DONOR FUNCTION OF THE PYRUVATE-FORMATE EXCHANGE SYSTEM*

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A rapid exchange between formate and the carboxyl group of pyruvate was first observed by Utter *et al.* in extracts of *Escherichia coli* E-26 (1, 2). These extracts also showed a rapid phosphoroclastic splitting of pyruvate to acetyl phosphate and formate. The formate incorporation into pyruvate was therefore interpreted to indicate a reversibility of the phosphoroclastic split.



Somewhat contrary to expectations, however, formate exchange was not enhanced by the addition to acetyl phosphate.

Further evidence for a reversibility of the phosphoroclastic reaction was gained by the use of carboxyl-labeled acetate (1), which, in the presence, but not in the absence of adenosine triphosphate (ATP), gave rise to carbonyl-labeled pyruvate. The latter reaction was, however, considerably slower than the formate incorporation. This was attributed to a probable slowness of the primary phosphorylation of acetate.

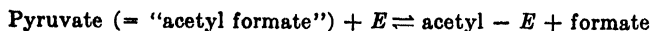
Recently, Strecker *et al.* reinvestigated the formate exchange with similar extracts of *E. coli* E-26 (3). They prepared radioactive acetyl phosphate and failed to obtain incorporation of its acetyl group into pyruvate, but confirmed the rapid formate exchange reported by Utter *et al.* (1). Their extracts, however, although obtained from the same original strain of *E. coli* E-26, differed in an important way from those used by Utter *et al.* The pyruvate metabolism in these extracts went almost exclusively through dismutation to lactate, acetyl phosphate, and CO_2 . Only a very weak phosphoroclastic reaction, if any, was observed. Apparently some change of metabolic patterns had taken place during the intervening years.

They could show, furthermore, that aged extracts which no longer reacted upon pyruvate still exhibited an active exchange between formate and the carboxyl group of pyruvate. Evidence was thus adduced for a

* This investigation was supported in part by a research grant from the National Cancer Institute, United States Public Health Service.

† Fellow of the Rockefeller Foundation.

new alternative type of formate exchange. In explanation, they proposed, among other possibilities, the existence of an equilibrium reaction between pyruvate, a C_2 -enzyme complex, and formate.



In the meantime, work in this laboratory had yielded considerable evidence that coenzyme A (Co A) was quite generally involved in acetyl transfer reactions (4), as, for example, in acetylation of aromatic amines (5) and in the synthesis of acetoacetate (6) and citrate (7). In all these cases, evidence had been obtained for a common acetyl donor system, presumably an acetyl-Co A-enzyme complex (8, 9).

It appeared, therefore, of considerable interest to determine whether the above exchange reaction also might involve Co A. If so, the acetyl-Co A-enzyme complex might then not only be formed from ATP + acetate in tissue extracts or from acetyl phosphate with bacterial "transacetylase" (8, 9), but also might be derivable directly from pyruvate. Such a possibility would then explain the type of reactions in which pyruvate apparently yielded active acetyl directly without any signs of intermediate formation of either acetate or acetyl phosphate.

Materials and Methods

Bacterial Extracts—*E. coli*, E-26, was grown as by Strecker *et al.* (3) in media containing 0.4 per cent beef extract, 0.4 per cent peptone, 0.2 per cent yeast extract, 0.2 per cent sodium chloride, and 10 per cent tap water. The cells were continuously aerated. After 16 to 18 hours of growth at 28–29°, the cells were harvested on a Sharples centrifuge and suspended once in water and recentrifuged. The cell paste was kept in the deep freeze at –30° to –35°, and samples were taken out at convenience for grinding. The paste was kept frozen for at least 2 weeks without loss of activity. Extracts were prepared by using essentially the glass grinding procedure of Utter *et al.* (1). For grinding small samples, it appeared, however, more convenient and not less efficient to grind the glass-paste mixture in a glass homogenizer of the Potter-Elvehjem type.

About 2 ml. of paste were mixed with an equal volume of glass powder and ground for about 20 minutes with a well cooled glass homogenizer. The ground mixture was triturated with 6 to 8 ml. of water and the glass was removed by centrifugation at low speed. The supernatant fluid was then centrifuged for 15 minutes at 12,000 r.p.m. in a Servall centrifuge in the cold room and a clear extract was obtained.

Treatment with Dowex-1—It has been found in this laboratory (9) that treatment with the anion exchange resin, Dowex-1, removed Co A conveniently and very effectively from enzyme solutions. The 70 mesh

Dowex-1 resin was first treated several times with *N* hydrochloric acid and then carefully washed acid-free with water. The neutral Dowex chloride resin was centrifuged, and the last drops of adhering water finally drawn off with a little filter paper. About one-half the volume of this semi-dry resin was mixed with a volume of bacterial extract and kept in an ice bath for 20 to 30 minutes. It was then centrifuged and eventually filtered through a small cotton plug. The pH of the solution after the Dowex treatment was about 6. The Dowex treatment generally resulted in a slight acidification of the protein solution, and, with an acid-sensitive enzyme, it is advisable to add a slight excess of base, starting, for example, with about pH 8, which prevents the pH from falling below 7.

Radioactive Material and Determination of Activity—Radioactive sodium formate was obtained as a solution from Oak Ridge,¹ and diluted with water to an activity of 200,000 c.p.m. per 0.1 ml. The formate content of this solution was negligible. Generally, 50 μ M of formate, with the 200,000 c.p.m. added to it, were used in a total volume of 1 ml., including enzyme solution. This corresponded to a specific activity of 4000 c.p.m. per μ M of formate. Specific activities throughout this paper are given in counts per minute per micromole.

For the determination of radioactivity, the pyruvate, generally 40 μ M, contained originally in 1 ml. of incubation mixture, was precipitated from the trichloroacetic acid filtrate as the dinitrophenylhydrazone. To remove active formate, the precipitate was washed on the centrifuge twice with 0.3 *N* HCl and then dissolved with a little 10 per cent carbonate solution, diluted to 2.5 ml., and reprecipitated with 0.2 ml. of 85 per cent formic acid and 0.3 ml. of 3 *N* HCl. The precipitate was washed with HCl and finally with water.

The dinitrophenylhydrazone was plated as a slurry. Approximately 1 mg. $\pm$ 20 per cent was transferred to a regular copper dish and dried by blowing hot air over it. For the counting, a Tracerlab Autoscaler was used with a thin window Geiger tube. With this uniform procedure of plating, no correction for self-absorption was applied. To determine the specific activity, the plated dinitrophenylhydrazone was dissolved with sodium carbonate solution and transferred to a colorimeter tube where it was read in 2 per cent sodium hydroxide after appropriate dilution.

A further localization of the radioactivity appeared unnecessary. Previous work (1, 3) had shown conclusively that in these extracts formate exchanges only with the carboxyl group of pyruvate.

Miscellaneous Methods—Acetyl phosphate was determined by the hydroxamic acid method (10). Sulfanilamide and coenzyme A were determined as described by Kaplan and Lipmann (11).

¹ Isotope Division, United States Atomic Energy Commission.

Effect of Coenzyme A on Incorporation of Formate into Pyruvate—The enzyme solution obtained in the manner described above proved suitable for the study of the formate exchange reaction. Pyruvate was quite stable in this solution. Only 2 to 4 per cent of 40 μM of pyruvate generally disappeared on incubation for 60 to 90 minutes at 29°, with 0.2 to 0.3 ml. of extract per ml. Occasionally somewhat more was metabolized, but, even then, the final concentration of pyruvate was never less than 85 per cent of the original. Exchange between pyruvate and formate in these extracts was found to be quite rapid, frequently leading in an hour or less to practically complete equilibrium.

Formate metabolism was not studied. However, judging from previous experience with this type of extract (1, 2), very little, if any, formate deg-

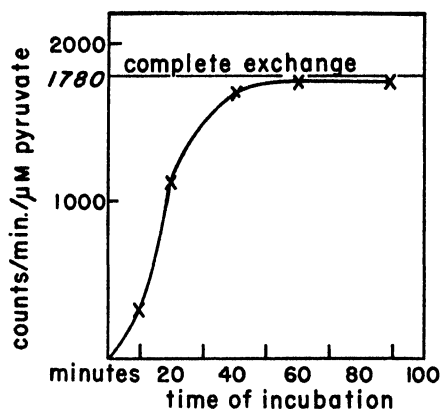


FIG. 1. Formate-pyruvate exchange. Standard experimental conditions as described in the text.

radation would be expected to take place, particularly when the extract is derived from aerated cells grown in the absence of glucose.

Throughout this study, a rather uniform experimental procedure was adopted. In a total volume of 1 ml. 0.2 to 0.3 ml. of bacterial extract was contained in 0.1 M phosphate buffer of pH 6.7. The samples were incubated at 29° in narrow test-tubes without removal of the air. 40 μM of sodium pyruvate were present and 50 μM of formate, with a total of 200,000 c.p.m. If the concentration of pyruvate as well as of formate remained unchanged, a maximum activity of pyruvate of 1780 c.p.m. per micromole would be obtained at equilibrium.

A representative experiment is shown in Fig. 1. A sample of Dowex-treated enzyme was used in this experiment which was complemented with 10 units of Co A. After 40 minutes incubation, almost complete, and

after 60 minutes, a complete equilibration was reached. The final figure for pyruvate was 1760 c.p.m. per micromole, and this value did not change after 30 minutes further incubation. It practically corresponded with the theoretically expected 1780 c.p.m. per micromole. A pyruvate determination was made at the end of the experiment, and showed that during incubation no determinable degradation of pyruvate had taken place.

The treatment with the anion exchange resin Dowex-1 uniformly caused a considerable decrease in activity. Occasionally a crude extract was found to be practically inactive without Co A and could be reactivated by addition of the coenzyme. Representative examples of reactivation by Co A are shown in Table I.

Saturation with Co A occurs at remarkably low levels. As little as 0.5 unit of Co A gives full activation. In the experiments shown in Fig. 2, two different coenzyme preparations were used. Dowex-treated enzyme

TABLE I

Reactivation of Formate Exchange Reaction by Co A

Experimental conditions as described in the text.

Enzyme preparation	Co A added	Activity of pyruvate	Incubation time
	<i>units per ml.</i>	<i>c.p.m. per μM</i>	<i>min.</i>
Crude Extract I	0	1520	30
	3	1520	
Dowex-treated Extract I	0	210	40
	3	1110	
Crude Extract II*	0	13	
	10	1275	

* Assay showed no determinable amount of Co A.

was incubated in the usual manner, and readings were taken after 20 minutes incubation. The data show that the two coenzyme preparations reactivated at approximately equivalent unit levels. Half activation was obtained with 0.1 unit. Our unit is defined as that amount which is necessary for half activation of sulfanilamide acetylation in a liver extract under comparable conditions (11). Co A showed, therefore, about a 10 times greater affinity here than in the sulfanilamide-acetylation system.

The Co A preparations used in these experiments were not pure substances, although, at least in some trials, the most highly purified Co A available was used. The chief contaminant in most of these preparations is adenylic acid and probably some adenylyl polyphosphate (12). Therefore, the effect of adenylic acid and ATP on the exchange reaction was studied. The experiments of Table II show that neither compound was an activator. On the contrary, the addition of 5 μ M of ATP caused a very definite in-

hibition. It should be mentioned here that the formate exchange system was found to be rather sensitive and easily inhibited by a variety of substances. Some Co A preparations showed an apparent maximum activity, owing obviously to inhibition at higher concentrations. Some such data

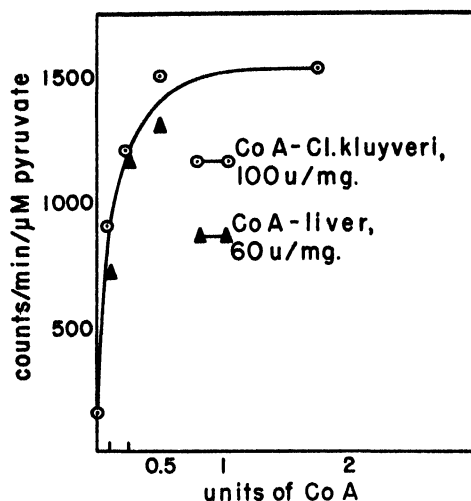


FIG. 2. Activation of the exchange reaction by Co A preparations obtained from *Clostridium kluveri* and from pork liver, respectively. A Dowex-treated extract was used. The readings were taken after 20 minutes incubation.

TABLE II

Effect of Adenylic Compounds on Exchange Reaction

Dowex-treated enzyme, 0.15 ml. per ml. of total volume; other conditions as described in the text.

Enzyme preparation No. (Dowex-treated)	Co A	5-Adenylic acid	ATP	Specific activity of pyruvate
	units per ml.	μM	μM	
3	10	5		1 825
4	10		5	30 26
	10		5	511 86

are presented in Table III. It was also found that degradation products of Co A obtained by treatment of Co A with fresh pigeon liver extract exerted a considerable inhibitory effect. In some experiments, collidine buffer of pH 6.7 was tried instead of phosphate. It was found that the

collidine buffer inhibited practically completely in the presence as well as in the absence of added phosphate. As will be mentioned later, cysteine is quite inhibitory.

Tests for Effect of Coenzyme A on Pyruvate Degradation—As mentioned before, our extracts degraded pyruvate little or not at all. In some of the more active extracts, the effect of Co A was tested and pyruvate disappearance as well as acetyl phosphate formation was found to be activated by Co A. A detailed study was not made to determine whether a dismu-

TABLE III

Inhibitory Effect of Higher Concentrations of Some Co A Preparations

Dowex-treated enzyme. Usual conditions, 45 minutes incubation at 30°, pH 6.7.

Co A preparation added, units per mg.	Co A added, c.p.m. per μM pyruvate		
	0 unit	5 units	10 units
None	193		
<i>C. kluyveri</i>		1580	1360
Liver Preparation W		1440	1080
“ “ L		1390	1410
“ “ P ₁		1440	1390

TABLE IV

Effect of Co A on Pyruvate Split and Acetyl Phosphate Formation

Experimental conditions as usual; Dowex-treated enzyme; 50 μM of pyruvate added. The pyruvate and acetyl phosphate data refer to two different experiments. Incubation time 60 minutes.

Preparation No.	Co A	Pyruvate disappeared	Acetyl phosphate formed
	units per ml.	μM	μM
1	0	1.7	
	25	7.1	
2	0		0.2
	15		1.1

tative or a direct phosphoroclastic split had occurred. The experiment recorded in Table IV seems to indicate that in the enzyme system of *E. coli* the phosphoroclastic split may be considerably activated by Co A. No definite conclusions as to the mechanism of this activation seem warranted at the present time.

In view of these observations, a few tests with extracts of *Lactobacillus delbrueckii* were carried out. A dry preparation made about 10 years ago was found to be still active. A phosphate extract was prepared (13) and treated with Dowex in the manner described for the *E. coli* extract. As

is shown in Table V, no effect of Co A addition on pyruvate oxidation or acetyl phosphate formation was found. The Co A content of this extract was determined, and barely determinable amounts were found, less than 0.4 unit per ml. The original extract, before Dowex treatment, was found to contain very little Co A, only about 1 unit per ml. Since it is known that the oxidation of the pyruvate in these extracts requires the presence of cocarboxylase as well as of flavin-adenine dinucleotide (13), the effect of the addition of these two coenzymes after Dowex treatment was also tested. As is shown in Table V, only a very small effect was observed. The Dowex treatment of this extract, therefore, removed only partially the cocarboxylase and flavin-adenine dinucleotide.

TABLE V

Test of Co A with Pyruvate Oxidation by Dowex-Treated L. delbrueckii

0.3 gm. of dry Preparation 1-3, 1939, of *L. delbrueckii* was extracted with 4.5 ml. of $M/15 Na_2HPO_4$ by shaking for 30 minutes at 37° . About 3 ml. of extract were obtained and treated with 0.5 volume of Dowex-1 as described. Each Warburg vessel contained 0.7 ml. of extract, 0.05 ml. of $M KH_2PO_4$, in a total volume of 1 ml. At the start, 0.1 ml. of 0.8 M sodium pyruvate was added from a side arm. Acetyl phosphate was determined by the hydroxamic acid method after termination of the experiment. Shaken with air for 90 minutes at 37° .

Co A, 20 units	Cocarboxylase, 30 γ	Flavin-adenine dinucleotide, 15 γ	O ₂ μM	Acetyl phosphat formed μM
—	—	—	—12.4	
+	+	+	—13.5	12.0
—	+	+	—13.0	12.2

In earlier experiments, it had been found that precipitation of pyruvic oxidase of *L. delbrueckii* with ammonium sulfate at pH 3 yielded a preparation which could be fully reactivated by addition of cocarboxylase and flavin-adenine dinucleotide (13, 14). Recent experiments in this laboratory with Dr. Earl Stadtman have shown that a similar treatment of extracts of *Clostridium kluyveri* completely removes Co A. In view of all these facts, it appears very unlikely, although it is not definitely excluded, that Co A participates in the oxidation of pyruvate in *L. delbrueckii*.

Direct Derivation of Active Acetyl from Pyruvate—The main purpose of this study had been the exploration of the question whether the pyruvate molecule might serve directly as an acetyl donor. The Co A dependence of the formate exchange, described above, aligns this reaction closely with acetyl transfer systems. In order to see whether it could serve as acetyl donor system, this reaction was now coupled with an acetyl acceptor enzyme.

It was reported recently by Chou *et al.* (8) that pigeon liver extracts could be separated into various acetyl acceptor fractions by acetone treatment in the cold. These acceptor fractions could then be recoupled with the tissue acetyl donor system as well as with microbial donor systems to yield acetyl transfer reactions. For the present test, the sulfanilamide acceptor enzyme was used as acceptor system. It was mixed with the *E. coli* extract. The results of such an experiment are presented in Table VI.

The data show that acetylation of sulfanilamide occurred with this enzyme combination when pyruvate was added. The extract contained only traces of phosphate and with this particular extract, even with an excess of phosphate, no appreciable acetyl phosphate formation was observed from pyruvate. It appears from Table VI that pyruvate is practically

TABLE VI

Sulfanilamide Acetylation with Pyruvate As Acetyl Donor

Freshly prepared, Dowex-1-treated *E. coli* extract was used. Samples were incubated in a total volume of 1 ml., containing, if added, 0.3 ml. of liver Fraction A-60 and 0.3 ml. of *E. coli* extract. All samples contained 10 μM of cysteine, 0.1 M tris(hydroxymethyl)aminomethane (Commercial Solvents) buffer of pH 7.5, and 0.5 μM of sulfanilamide. The extract contained not more than 6.10^{-4} M phosphate. 90 minutes incubation; temperature 37°.

Enzyme mixture	Pyruvate	Acetyl phosphate	Sulfanilamide acetylated
	μM	μM	μM
<i>E. coli</i> + liver	25		0
" " + " + Co A. . . .	25		0.40
" " + " + " " . . .			0.05
" " + " + " " . . .		12	0.43
" " + " + " " . . .	25	12	0.48

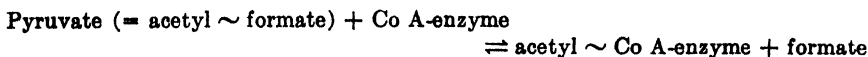
equivalent in this system to acetyl phosphate as an acetyl donor. The extract exhibited good activity with added acetyl phosphate as an acetyl donor, indicating the presence of phosphotransacetylase (8, 9).

Separate experiments on the formate exchange reaction with pyruvate, under similar conditions of pH and in the presence of cysteine, revealed that the conditions used in the experiment of Table VI were not optimum for exchange. At pH 7.5, which is optimum for sulfanilamide acetylation, the rate of the formate exchange was about 20 to 25 per cent of that at pH 6.7, which had been used in most of the exchange experiments described here. An additional inhibition was caused by cysteine addition, which is essential for the functioning of the acceptor enzyme. Even under these conditions, however, the pyruvate-formate exchange system yielded enough

acetyl-Co A-enzyme complex to bring about the acetylation of sulfanilamide.

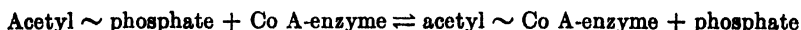
DISCUSSION

The results reported by us suggest a reformulation of the formate exchange reaction with the inclusion of Co A.



In a reversible manner, the acetyl part of pyruvate becomes joined in energy-rich linkage to the Co A-enzyme and formate is liberated. In the reverse reaction, formate rejoins the enzyme-Co A $\sim$ acetyl to reform pyruvate.

The formate exchange reaction is quite analogous to the phosphate exchange between acetyl-bound and inorganic phosphate in microbial extracts (2, 15), for which a similar interpretation had been proposed by Stadtman and Barker (15). The dependence (9) of the acetyl phosphate-phosphate exchange on Co A makes the analogy complete.



The enzyme catalyzing the above reaction has been identified with the microbial factor which makes acetyl phosphate available as an acetyl donor (8). It is referred to as transacetylase, or, more specifically, phosphotransacetylase. It appears from the present results that in *E. coli* there exists, besides the phosphotransacetylase, a formotransacetylase which makes pyruvate ("acetyl formate") available as an acetyl donor.

The proposed formulation brings out the parallel between the two reactions. Both lead to the formation of an acetyl-Co A-enzyme complex. Although the enzyme on which the complex forms is not identical in these two cases, it obviously delivers active acetyl to acetyl acceptor systems. So far it has not been possible to decide whether a dissociable acetyl-Co A exists or whether the acetyl is transferred directly from an acetyl-Co A-enzyme complex.

SUMMARY

The exchange of formate with the carboxyl group of pyruvate in extracts of *Escherichia coli* E-26 (Strecker *et al.* (3)) is found to be a coenzyme A-catalyzed reaction. Co A is removed from these extracts by treatment with the anion exchange resin Dowex-1. The treated extracts become nearly or fully inactive. Activity is regained on addition of Co A. The saturation of this system occurs at the rather low Co A concentration of 0.5 unit per ml.

It is proposed that in this reaction an acetyl $\sim$ Co A-enzyme complex is formed intermediately and it is shown that this enzyme system can serve as acetyl donor to sulfanilamide when coupled with the acetyl acceptor fraction for sulfanilamide (8) from pigeon liver. Through mediation of this system, pyruvate appears to act as an immediate acetyl donor.

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EFFECT OF EPINEPHRINE ON RAT DIAPHRAGM

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The specific effect of epinephrine on muscle consists of an accelerated breakdown of glycogen which results in an accumulation of hexose-6-phosphate and a corresponding decrease in inorganic phosphate. This is most clearly seen in experiments with isolated frog muscle in which a secondary increase in the rate of lactic acid formation is not a prominent feature (1). In intact mammals the accumulation of hexose-6-phosphate in muscle is accompanied by an outflow of lactic acid from muscle.

Another effect of epinephrine which has received attention is a decreased rate of uptake of glucose in muscle (2). This has recently been reaffirmed by Somógyi (3) in experiments on man. The question has been raised whether this is a direct effect of epinephrine or whether it is mediated indirectly through increased anterior pituitary secretion. That injection of epinephrine elicits the secretion of adrenotropic hormone from the anterior pituitary has been clearly demonstrated (4). Cohen (5) concluded that the effect was an indirect one, since injection of epinephrine caused decreased anaerobic glycolysis in diaphragms from normal but not from hypophysectomized rats.

In the present paper are reported experiments on carbohydrate balance on rat diaphragm under aerobic conditions; they show that addition of epinephrine *in vitro* has a marked effect on glucose uptake, glycogen formation, and lactic acid production. As a necessary preliminary to this work the determination of micro quantities of glycogen has been reinvestigated.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain were used. They were fasted for 24 hours prior to removal of the diaphragm. The suspension medium consisted of 0.01 M phosphate-Ringer's solution, pH 7.4, and contained 140 mg. per cent of glucose initially. Experimental conditions were so arranged that the glucose concentration after incubation did not fall below 100 mg. per cent.

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The diaphragms were immediately immersed in a large volume of ice-cold medium and shaken for 20 to 30 minutes in order to remove preformed lactic acid (see below). From each hemidiaphragm one-third was taken for glycogen determination. After the preliminary washing period, four pieces of diaphragm from two animals (weight about 200 mg.) were transferred to a Warburg vessel containing 2 ml. of medium. The vessels contained an alkali well with filter paper for the absorption of CO_2 ; they were attached to manometers and gassed with oxygen while remaining in an ice bath. After a 10 minute equilibration period at 37° , manometer readings were started and continued for 1 hour. After incubation the vessels were detached, stoppered, and chilled in an ice bath before samples were taken. Aliquots of the medium were analyzed for glucose (6) and lactic acid (7), while the diaphragm was analyzed for glycogen. After removal of the diaphragms and medium the vessels were again attached to manometers and the CO_2 trapped in the alkali well was displaced by tipping in acid. Separate experiments were run to determine the amount of CO_2 present in the diaphragms and medium at zero time and at the end of incubation, and this was applied as a correction in the calculation of metabolic CO_2 production.

Epinephrine (Parke, Davis and Company, 1:100 solution) was diluted with ice-cold medium immediately before the experiment so that the final concentration was 1 γ per ml. (or 2 γ in each vessel). In some experiments rats were injected subcutaneously with 0.02 mg. of epinephrine per 100 gm. and the diaphragms removed 1 hour later.

Glycogen Determination—Only a few of the numerous control experiments will be presented. The main point to be considered in a micro-procedure is that glycogen has a definite solubility in 50 to 60 per cent alcohol, about 5 to 10 γ per ml. according to Kerly (8). Consequently, if the total amount of glycogen to be determined is of the order of 100 γ serious losses will occur by precipitating glycogen from too large a volume or by carrying out reprecipitations (in order to purify glycogen). The amount of glycogen lost under these conditions was determined as follows: The glycogen content of KOH hydrolysates of rat muscle was determined by the macroprocedure of Good, Kramer, and Somogyi (9) in samples containing at least 1 mg. of glycogen. Four aliquots of these hydrolysates, containing from 65 to 165 γ , average 114 γ , of glycogen, were each precipitated twice in a total volume of 5 ml. of 60 per cent alcohol as in the macro-procedure. Extra salt was added as an aid in the precipitation of glycogen. The average loss of glycogen was 51 per cent or 5.8 γ per ml. of 60 per cent alcohol. Six other samples, containing an average of 100 γ of glycogen, were each precipitated three times in 0.7 ml. of 60 per cent alcohol, extra salt being added each time. The average loss of glycogen was 14 per cent

or 6.2 γ per ml. of 60 per cent alcohol. In both cases the loss corresponds to the solubility of glycogen in alcohol reported by Kerly.

On the basis of these findings, the following procedure was adopted, which is described with omission of some details. Diaphragms (100 to 200 mg.) were digested for 30 minutes at 100° in 0.2 to 0.3 ml. of 30 per cent KOH in micro centrifuge tubes; 0.1 ml. of 2 per cent Na_2SO_4 was added, followed by alcohol to a final concentration of 65 to 70 per cent. Thin glass rods were used for thorough mixing. After standing overnight, the glycogen was removed by centrifugation, the supernatant fluid decanted, and the inverted tubes drained on filter paper. The walls and precipitate were washed once with 0.5 ml. of 65 per cent alcohol in order to remove non-fermentable reducing substances. The last traces of alcohol were expelled by evaporation in a water bath at 70°, the precipitate was dissolved in 0.4 ml. of 1 N H_2SO_4 , and the tubes covered. After hydrolysis for 3 hours at 100°, the contents were neutralized with 1 N NaOH to pH 7.4, with a standard amount of phenol red as internal indicator. Aliquots were taken for glucose determinations by the Nelson (6) method.

In five determinations with this method on KOH hydrolysates of muscle, covering a range of 65 to 160 γ , average 130 γ , of glycogen, the standard error was ± 2.1 per cent when compared with the macroprocedure. In eight determinations by the microprocedure on the same KOH hydrolysate, the mean glycogen content per 100 gm. of tibialis muscle was 459 mg., with a standard error of ± 1.2 per cent. Finally, 100 γ of glycogen were added as an internal standard to KOH hydrolysates of muscle of known glycogen content; the average amount recovered was 98.6 per cent with one precipitation in a volume of 0.7 ml. and 87 per cent with three precipitations, each in a volume of 0.7 ml.

Lactic Acid Determination—Diaphragms were ground in 5 per cent ice-cold trichloroacetic acid and the filtrate analyzed by the method of Barker and Summerson (7). Six diaphragms analyzed immediately after death contained an average of 74 ± 19 mg. of lactic acid per 100 gm. of muscle. Removal of diaphragm under amytal anesthesia did not result in lower values. Apparently a loss of glycogen cannot be avoided during excision of muscle, no matter how rapidly and carefully it is done. In addition to lactic acid, one may also expect an increase in hexose-6-phosphate as the result of glycogenolysis.

The preformed lactic acid was removed by shaking the diaphragms for 20 to 30 minutes in several changes of ice-cold medium; this reduced the average lactic acid content of the diaphragms to 11 mg. per cent. If the preformed lactic acid is not removed, it diffuses into the medium during incubation, making it appear as if twice as much lactic acid had been formed as was actually the case. During incubation the ratio of tissue to

medium was about 1:10, and it was therefore sufficient to analyze the medium for lactic acid in order to calculate the rates of production.¹

Results

Normal Diaphragms—Control values were obtained on a group of younger (100 gm.) and of older (200 gm.) rats (Table I). In both groups

TABLE I

Effect of Epinephrine on Carbohydrate Balance in Isolated Diaphragm

The concentration *in vitro* was 2 γ of epinephrine in 2 ml. of medium. The injected rats received 0.02 mg. of epinephrine per 100 gm. 1 hour before removal of the diaphragm. Incubation period, 1 hour at 37°. Average values and standard errors recorded.

Diaphragm from	Treatment	No. of experiments	O ₂ consumption	R. Q.	Glucose uptake	Glycogen formed	Lactic acid formed	Glucose accounted for
			ml. per gm. wet tissue per hr.		mg. per gm. wet tissue per hr.	per cent*	per cent*	per cent*
100 gm. rats	Controls	16	1.7 $\pm$ 0.07	0.92	2.8 $\pm$ 0.13	+50 $\pm$ 6†	24 $\pm$ 2	74
	Epinephrine <i>in vitro</i>	10	1.5 $\pm$ 0.04	0.89	1.9 $\pm$ 0.14	-13 $\pm$ 5	52 $\pm$ 5	39
	Epinephrine injected	10	1.9 $\pm$ 0.06	0.90	3.3 $\pm$ 0.17	+38 $\pm$ 5‡	17 $\pm$ 1	55
200 gm. rats	Controls	8	1.3 $\pm$ 0.06	0.89	2.4 $\pm$ 0.09	+56 $\pm$ 9	23 $\pm$ 2	79
	Epinephrine <i>in vitro</i>	7	1.5 $\pm$ 0.07	0.85	1.8 $\pm$ 0.11	-9 $\pm$ 8	56 $\pm$ 6	47

* Per cent of glucose uptake.

† Average glycogen before and after incubation, 139 and 280 mg. per cent, respectively.

‡ Average glycogen before and after incubation, 27 and 149 mg. per cent, respectively.

about 50 per cent of the glucose taken up from the medium during 1 hour of incubation was deposited as glycogen, while about 24 per cent was converted to lactic acid.² The oxygen consumption (at a respiratory quotient of 0.9) was more than sufficient to account for the oxidation of the remainder of about 25 per cent. Two groups of workers (10, 11) measured

¹ Determination of the lactic acid concentration in the diaphragms after incubation, whether or not they had undergone preliminary washing, indicated approximate diffusion equilibrium with the medium.

² The fact that the isolated diaphragm forms lactic acid under aerobic conditions indicates that it is not being maintained *in vitro* under strictly physiological conditions.

the amount of radioactive CO_2 formed (from C^{14} -labeled glucose in the medium) and found an oxidation corresponding to about 7 per cent of the glucose uptake. Preformed lactic acid (a readily oxidizable substrate) was not removed in their experiments and glycogen formation accounted for 11 to 30 per cent of the glucose uptake. Gemmill (12) accounted for about 60 per cent of the glucose uptake in terms of glycogen.

Effect of Epinephrine in Vitro—The addition of epinephrine *in vitro* produced the following effects, which were all highly significant statistically. The glucose uptake was diminished, the lactic acid formation was increased, and there was a negative glycogen balance. Riesser (13) had observed previously that the addition of 2 to 5 γ of epinephrine per ml. of a medium containing glucose caused a loss of glycogen instead of the gain observed in the control diaphragms.

TABLE II

Relation of Lactic Acid Formation to Accumulation of Intermediary Products

Calculated from Table I. The values of Column 1 have not been corrected for the amount of glucose oxidized; see the text.

Experimental conditions	Glucose uptake less glycogen formed (1)	Lactic acid formed (2)	Ratio (2):(1) (3)
	<i>mg. per gm. per hr.</i>	<i>mg. per gm. per hr.</i>	
Controls*.....	1.4	0.67	0.48
Epinephrine <i>in vitro</i> *.....	2.1	0.99	0.47
Control†.....	1.1	0.56	0.51
Epinephrine <i>in vitro</i> †.....	2.0	1.01	0.50

* Diaphragms from 100 gm. rats.

† Diaphragms from 200 gm. rats.

The glucose accounted for was only 39 to 47 per cent compared to 74 to 79 per cent for the controls (Table I). Preliminary measurements by an enzymatic method indicate that the diaphragms treated with epinephrine contain more hexose-6-phosphate than the controls. Such increases as have been observed previously in isolated frog muscle and in muscle of rats injected with epinephrine (of the order of 0.8 mg. per gm., as hexose) would be sufficient to account for the glucose deficit. That a larger amount of intermediary products accumulates in the diaphragms treated with epinephrine than in the controls can be shown by calculating the difference, glucose uptake minus glycogen formed (Table II).³ The in-

³ The values recorded in Column 1, Table II, should be decreased by the amount of glucose oxidized, roughly 0.7 mg. per gm. per hour, equivalent to about 35 per cent of the total O_2 consumption. Omitting this correction seemed permissible, since the O_2 consumption and the R. Q. did not change significantly when epinephrine was added (Table I).

creased rate of lactic acid formation in the diaphragms treated with epinephrine appears to be proportional to the accumulation of intermediary products, as shown in Column 3 of Table II, and is therefore regarded as secondary to the glycogen breakdown.

If, as seems probable, the primary effect of epinephrine is on the phosphorylase system, it will have to be explained how the direction of activity of this enzyme is reversed. The decrease in glucose uptake, some 30 per cent, although statistically significant ($P < 0.001$), is not large enough to account for the fact that glycogen is being broken down instead of being built up.

The lactic acid concentration in the medium was higher and consequently the pH change somewhat greater when epinephrine was added. In order to see whether this was responsible for the decreased glucose uptake 300 mg. of normal diaphragm (instead of the usual 200 mg.) were incubated in 2 ml. of medium. This resulted in similar final lactic acid concentrations in the medium to those in the epinephrine experiments (about 10 mg. per 100 ml.) and in similar pH changes (from 7.3 before to 6.9 after incubation), but the rate of glucose uptake was not affected. Further work is needed to establish the nature of the effect of epinephrine on glucose uptake. The report of Meyerhof and Randall (14) that adrenochrome inhibits hexokinase in brain extract is of particular interest in this connection.

Effect of Epinephrine Injection—Results obtained with diaphragms removed 1 hour after epinephrine injection are shown in Table I. The increase in glucose uptake (mg. per gm. per hour, control *versus* injected, $P = 0.03$) and the decrease in lactic acid formation (mg. per gm. per hour control *versus* injected, $P = 0.06$) were of marginal significance. The decrease in lactic acid formation in per cent of glucose uptake ($P = 0.003$) was of somewhat greater statistical significance. The most marked effect of injection was a depletion of diaphragm glycogen (Table I); this corresponds to the well known effect of epinephrine on muscle glycogen *in vivo*. In spite of the low initial content, the gain in glycogen during incubation (+1.22 mg. per gm. per hour) was not different from that observed in the controls (+1.41 mg. per gm. per hour).

It seems unlikely that significant amounts of epinephrine are present in diaphragms removed 1 hour after injection of 0.02 mg. per 100 gm. of rat, partly because epinephrine is rapidly destroyed in the tissues, partly because of the large dilution and washing of the diaphragm prior to incubation. If the injection of epinephrine caused a change in the normal balance between insulin, which increases, and an anterior pituitary factor, which decreases, glucose uptake, such a change might have been detected, since it has been shown by Stadie *et al.* (15) that these hormones are not easily

removed by washing the diaphragm. The results obtained with diaphragms from rats 1 hour after injection show a slight shift in the balance in favor of insulin. It is possible, however, that the time interval chosen was too long to detect increased pituitary secretion. Adrenotropic hormone is known to be released after epinephrine injection (4), but it is not the pituitary substance that causes decreased glucose uptake by rat diaphragm. The results as a whole suggest that the decreased glucose uptake in muscle under the influence of epinephrine, observed previously *in vivo* and now also *in vitro*, is in part at least to be attributed to a direct effect on muscle.

SUMMARY

1. Carbohydrate balances in normal rat diaphragm showed that 50 per cent of the glucose taken up from the medium during 1 hour of incubation was deposited as glycogen, while 25 per cent was converted to lactic acid. The oxygen consumption, at a respiratory quotient of 0.9, was of sufficient magnitude to account for the oxidation of the remainder of 25 per cent. Methods for the determination of microgram quantities of glycogen were reexamined. It was found necessary to remove preformed lactic acid from diaphragms prior to incubation in order to obtain correct values for the rate of lactic acid formation.

2. Addition of epinephrine *in vitro* (1 γ per ml. of medium) caused a significant decrease in glucose uptake and increase in lactic acid formation. Instead of glycogen deposition there was a net loss of glycogen. The recovery of glucose was less than in the controls, owing to the accumulation of intermediary products.

3. Diaphragms removed from rats 1 hour after injection of epinephrine showed a depletion of glycogen. Glucose uptake was slightly increased and lactic acid formation decreased, while glycogen deposition was not significantly different from that found in the controls. The question of a direct *versus* an indirect effect of epinephrine on glucose uptake in muscle has been discussed.

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STUDIES OF THE METABOLISM OF TRYPTOPHAN LABELED WITH N^{15} IN THE INDOLE RING*

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Recent interest in tryptophan metabolism has been stimulated by evidence that tryptophan is a precursor of nicotinic acid in mammals (1) as well as in *Neurospora crassa* (2). The formation of kynurenine, kynurenic acid, and xanthurenic acid from tryptophan has been demonstrated by numerous experiments (3-5) and has been confirmed by isotope technique in the case of kynurenine and kynurenic acid (6). Recently it has been shown that indole plus serine can act as a precursor of tryptophan in *N. crassa*, and there are indications that the same relationship may hold true for certain bacteria (7). In order to study these and other aspects of tryptophan and indole metabolism, indole labeled with N^{15} and tryptophan labeled with N^{15} in the indole ring were synthesized.

EXPERIMENTAL

Synthesis of Indole Containing N^{15} —Potassium phthalimide (containing excess N^{15}) was converted successively into anthranilic acid (8), phenylglycine-*o*-carboxylic acid (9), indoxyl, and indole (10). The indole was recrystallized twice from ligroin. It contained 33.1 per cent excess N^{15} ; m.p. 52°; calculated N (based on 33.1 per cent N^{15}) 12.11 per cent; found, 12.22.

Synthesis of Tryptophan Containing N^{15} in Indole Ring— N^{15} -labeled indole was converted by way of gramine to DL-tryptophan by the method of Howe *et al.* (11). The over-all yield from potassium phthalimide was

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4.1 per cent of the theoretical amount. It contained 31.2 per cent excess N^{15} ; m.p. 286–290°; calculated N (based on 31.2 per cent N^{15}) 13.86 per cent; found, 13.86.

The tryptophan was resolved by the method of Shabica and Tishler (12); L-tryptophan, $[\alpha]_D^{20} = -31.5^\circ$; D-tryptophan, $[\alpha]_D^{20} = +31.6^\circ$ for 1 per cent aqueous solution.

Relationship of Tryptophan to Kynurenine, Kynurenic Acid, and Xanthurenic Acid—The rats and rabbits used in experiments on the conversion of tryptophan to kynurenic acid were fed a stock diet. Those used in experiments on the conversion of tryptophan to kynurenine were fed a polished rice diet for 2 to 3 weeks. Rats for experiments on the conversion of tryptophan to xanthurenic acid were fed a pyridoxine-deficient diet (13) for 4 to 5 weeks and injected with 1.5 mg. of desoxypyridoxine along with the tryptophan. In all cases the N^{15} -tryptophan was injected intraperitoneally in neutral solution. The level of tryptophan injected into rabbits ranged from 0.5 to 0.8 gm. per kilo of body weight, while for rats it ranged from 1.5 to 2.0 gm. per kilo of body weight. In the rabbit experiments, one animal was used; in those with rats, three to six animals were employed.

Kynurenine sulfate was isolated by the method of Kotake and Iwao (3) from the urine of rats and rabbits. Crystallization by addition of alcohol to the concentrated urine produced a substance which after two recrystallizations from 60 per cent alcohol had an N content of 8.48 per cent. While this is in agreement with the value of 8.51 per cent obtained by Kotake and Iwao (3) and of 8.54 per cent reported by Jackson and Jackson (14), it does not conform with the theoretical value of 9.15 per cent required by the revised formula of Butenandt *et al.* (15). However, when subjected to precipitation with mercuric sulfate in 0.3 N sulfuric acid, the product contained 9.12 per cent N, $[\alpha]_D^{20} = +7.4^\circ$ (1 per cent aqueous solution); reported $[\alpha]_D^{20} = +7.3^\circ$. The concentrations of N^{15} in the urinary kynurenine are given in Table I, Experiments 2, 6, and 7.

Kynurenic acid was isolated by the Capaldi method (16) from the urine of rats and rabbits. After two recrystallizations from 50 per cent acetic acid, the isotope content was constant. The melting point of kynurenic acid was 287°; reported 290° (17); of methyl ester, 224°; reported 224° (18). Concentrations of N^{15} in the urinary kynurenic acid are given in Experiments 1 to 4 and 7, Table I.

Xanthurenic acid was isolated from the urine of rats. Isolation was accomplished by strongly acidifying the urine with hydrochloric acid, allowing the precipitate to settle for 1 hour, and shaking the entire mixture with three portions of isobutanol. The isobutanol extract was dried, filtered, and evaporated to dryness *in vacuo*. The residual red-brown tar

was taken up in saturated sodium chloride solution and filtered. The insoluble residue was washed with water and then with acetone, dissolved in warm dilute sodium hydroxide solution, and reprecipitated with hydrochloric acid. The crude xanthurenic acid was purified as the methyl ester

TABLE I
N¹⁵ Excretion after Injection of Tryptophan and Kynurenine

Ex- peri- ment No.	Animal	Compound injected	N ¹⁵ con- centra- tion of compound injected*	Compound isolated	N ¹⁵ con- centra- tion of com- pound isola- ted*	N, Dumas analysis	
						Calcu- lated†	Found
						per cent	per cent
1	Rabbit	66.4 mg. DL-trypto- phan‡ + 1.021 gm. normal L-trypto- phan	1.071§	Kynurenic acid	0.850	7.41	7.55
2	"	201.0 mg. DL-trypto- phan‡ + 1.980 gm. normal L-trypto- phan	1.507§	Kynurenine sulfate Kynurenic acid	1.294 1.211	9.15 7.41	8.73 7.38
3	"	L-Tryptophan	0.248	Kynurenic acid	0.219	7.41	7.28
4	Rat	"	0.310	" "	0.287	7.41	7.44
5	"	"	0.310	Xanthurenic acid methyl ester	0.297	6.40	6.66
6	"	"	0.544	Kynurenine sulfate	0.504	9.15	9.22
7	"	18.3 mg. DL-trypto- phan‡ + 1.192 gm. normal L-trypto- phan	0.238§ 0.472	" " Kynurenic acid	0.486 0.455	9.15 7.41	8.98 7.23
8	"	Kynurenine sulfate, natural isomer	0.126	Xanthurenic acid	0.137	6.83	7.14
9	Rabbit	" "	0.172	Kynurenic acid	0.127	7.41	7.54

* All N¹⁵ values for tryptophan are based on the ring nitrogen only; those for kynurenine are based only on the nitrogen attached to the ring.

† Discrepancies due to isotope content are negligible.

‡ 31.2 per cent excess N¹⁵ in the ring.

§ Based on L isomer only.

|| Based on L + DL.

(19) or recrystallized by dissolving in slightly alkaline 50 per cent alcohol, heating, and acidifying. The xanthurenic acid melted at 288°; m.p. 290° (Lepkovsky *et al.* (19)). The methyl ester melted at 261–262°; m.p. 260–261° (19). The concentration of N¹⁵ in the urinary xanthurenic acid is given in Table I, Experiment 5.

Relationship of Kynurenine to Kynurenic Acid and Xanthurenic Acid—

A sample of N^{15} -labeled kynurenine sulfate was prepared from the urine of rats and rabbits which had received isotopic tryptophan, and purified by recrystallization from 60 per cent alcohol. After neutralization it was injected intraperitoneally into a rabbit fed a stock diet. The N^{15} concentration of the urinary kynurenic acid is given in Table I, Experiment 9.

Xanthurenic acid was obtained from the urine of three rats injected intraperitoneally with N^{15} -labeled kynurenine as given in Table I, Experiment 8.

Relationship of Tryptophan to Hemin—Each of three 250 gm. male rats on a tryptophan-deficient diet was injected in two doses with a total of 305 mg. of DL-tryptophan containing 31.2 per cent N^{15} . The rats were killed by exsanguination 17 days after the last dose of tryptophan, and the hemin was isolated (20) and recrystallized. It contained 0.014 per cent N^{15} excess.

Distribution of N^{15} after Ingestion of Labeled Indole, L-Tryptophan, D-Tryptophan, and DL-Tryptophan—Four young, mature rats were placed on a protein-free diet for 5 days and then fed the test compound mixed with their food. Rat 1 received 50 mg. of indole containing 33.1 per cent N^{15} excess, plus 400 mg. of DL-serine; Rat 2, 110 mg. of L-tryptophan containing 6.00 per cent N^{15} excess in the indole ring; Rat 3, 200 mg. of D-tryptophan containing 6.08 per cent N^{15} excess in the indole ring; and Rat 4, 140 mg. of DL-tryptophan containing 31.2 per cent N^{15} excess in the indole ring.

Urine and feces were collected during the period of consumption of the test compound (20 to 30 hours) and 24 hours thereafter (Urine 1 and Feces 1). Collection was continued for an additional 24 hours (Urine 2 and Feces 2). The rat was then killed and the carcass used for isolations.

Ammonia and urea were isolated only from Urine 1. Ammonia was isolated by adsorption on permutit. Urea for N^{15} determination was isolated as the xanthidrol derivative. Total urea nitrogen was determined by treating an aliquot of the ammonia-free filtrate from permutit with urease and adsorbing the liberated ammonia on permutit, followed by distillation and titration.

Tryptophan was isolated from the entire rat carcass as acetyl-DL-tryptophan as follows: After removal of the contents of the stomach and intestines, the entire rat carcass was hydrolyzed in 750 ml. of 5 N sodium hydroxide on a steam bath for 20 hours in an atmosphere of nitrogen. After filtration, the filtrate was cooled and made slightly acid with 18 N sulfuric acid. It was again filtered, diluted to 2.5 liters, and made 1 N with sulfuric acid. To this was added a solution of 60 gm. of mercuric sulfate in 1.5 liters of 1 N sulfuric acid. After the mixture had been heated to 60° on a steam bath and allowed to stand for 1 or 2 hours, the supernatant liquid was decanted and the voluminous precipitate recovered by centri-

fugation. The precipitate was finely suspended in 4 liters of 0.1 N sulfuric acid, thoroughly stirred, recovered by decantation and centrifugation, suspended in 600 ml. of water, and saturated with hydrogen sulfide 6 hours with vigorous stirring. The mercuric sulfide was removed, the filtrate was adjusted to pH 6, and a solution of 8 gm. of silver lactate in water added with stirring. After a few minutes the precipitate was removed by filtration, washed thoroughly, and discarded. This removed the purines and some dark colored material. Silver was removed as the sulfide and the tryptophan was reprecipitated from a volume of 4 liters with 60 gm. of mercuric sulfate in 1 N sulfuric acid. The lemon-yellow precipitate was washed with 0.1 N sulfuric acid and with water, and the mercury was removed as the sulfide. After filtration, the solution was evaporated to about 50 ml. *in vacuo*, neutralized with sodium hydroxide, evaporated to about 20 ml., made alkaline to phenolphthalein, and treated with excess of acetic anhydride, the solution being kept alkaline. It was finally made acid to Congo red and chilled. Crystallization, aided by seeding or scratching, was usually complete in 2 or 3 days. The acetyl-DL-tryptophan, after two or three recrystallizations from boiling water, melted at 205–206° (21).

The amino acids tyrosine, leucine, arginine, glutamic acid, and aspartic acid were isolated in the usual manner (22) from the combined liver, plasma, kidneys, and spleen of the rat receiving DL-tryptophan.

The results of the feeding experiments are shown in Tables II and III.

DISCUSSION

Relationship of Tryptophan to Kynurenine, Kynurenic Acid, and Xanthurenic Acid (Table I, Experiments 1 to 3)—As can be seen from these experiments, the N^{15} concentrations of kynurenine and kynurenic acid isolated from rabbit urine are approximately the same after injection of either L- or DL-tryptophan. There is no evidence of the utilization of D-tryptophan by rabbits under the conditions of this experiment for the formation of kynurenine and kynurenic acid. The N^{15} concentrations of the isolated kynurenine and kynurenic acid indicate that over 80 per cent of these substances originated from the injected tryptophan.

Experiments 4 to 6—The conversion of L-tryptophan to kynurenine, kynurenic acid, and xanthurenic acid in rats occurred with a very slight dilution, indicating that over 90 per cent of these substances originated from the injected tryptophan. The dilution in rats was considerably less than in rabbits; however, the rats received doses of tryptophan per unit of body weight approximately 3 times as high as for the rabbits.

Experiment 7—The results suggest that the rats converted the small amount of D-tryptophan present into L-tryptophan and utilized it in the

production of kynurenine and kynurenic acid. This inversion has been known in rats, since the D isomer is equal to the L isomer in growth promotion (23).

Experiments 8 and 9— N^{15} -Kynurenine injected into rats produced xanthurenic acid without dilution of the isotope (Experiment 8). N^{15} -Kynurenine injected into a rabbit produced kynurenic acid in which the isotope was significantly diluted (Experiment 9). It would therefore appear that kynurenine is normally formed in the rabbit and is not merely a means of getting rid of large excesses of tryptophan.

Relationship of Tryptophan to Hemin—The value of 0.014 per cent N^{15} excess in hemin isolated from rats indicates that there is no significant conversion of the ring structure of tryptophan to hemin in rats.

Distribution of N^{15} after Ingestion of Labeled Indole, L-Tryptophan, D-Tryptophan, and DL-Tryptophan—After ingestion of indole the very high N^{15} values of the whole Urine 1 and the low values of the ammonia and urea fractions indicate that the ring of ingested indole is only slightly disrupted and that the major portion is excreted as a compound in which the indole nucleus is intact, probably indican. The urinary excretion of isotope is almost complete 24 hours after ingestion. Fecal excretion is small, indicating great efficiency of absorption of indole by the gut (Rat 1, Table II).

After ingestion of L-, D-, and DL-tryptophan, the N^{15} values of urinary ammonia and urea are high and indicate that these products account for 11 to 18 per cent of the ingested N^{15} (Rats 2, 3, and 4, Table II).

The fact that in all cases the urinary ammonia has an N^{15} concentration 4 times greater than that of the urinary urea suggests the existence in rat kidney, as well as in liver, of an enzyme system which splits the indole nucleus of tryptophan to ammonia, which is excreted before conversion to urea. This behavior is unlike that of L- α -amino acids; which yield urea and ammonia of approximately equal isotopic concentrations (24), but resembles the behavior of D-leucine (25). In this case, a ratio of ammonia to urea of approximately 4:1 exists and is explained by the presence in rat kidney of a D-amino acid oxidase, which splits off the α -amino nitrogen as ammonia, which is rapidly excreted before conversion to urea.

In the experiment with DL-tryptophan, the rat received tryptophan with sufficiently high isotope concentration to provide a significant N^{15} concentration in the amino acids of the organs whose proteins undergo rapid turnover; i.e., liver, plasma, gut, and spleen (Table III). The N^{15} distribution in these amino acids follows the usual pattern (26), with the amidine group of arginine highest, glutamic and aspartic acids slightly lower, and leucine, ornithine, and tyrosine low. This demonstrates that the indole nitrogen of tryptophan can enter the general nitrogen pool.

TABLE II
Distribution of N¹⁵ after Ingestion of Labeled Indole and Tryptophan

Fraction	N ¹⁵	Total N	Per cent of isotope*
Rat 1, male, 164 gm.; fed 50 mg. indole containing 33.1% N ¹⁵ excess + 400 mg DL-serine			
	<i>per cent excess</i>	<i>mg.</i>	
Urine 1.	1.270	101.6	63.5
1, ammonia	0.026		
1, urea	0.041		
2.	0.42	48.0	1.0
Feces 1	0.87	87.6	3.8
2	0.47	15.0	0.3
Total			68.6
Rat 2, male, 158 gm.; fed 110 mg. L-tryptophan containing 6.00% N ¹⁵ excess in indole ring			
Urine 1.	0.244	103.4	55.5
" 1, ammonia	0.266	12.2	(7.1)
" 1, urea	0.067	65.1	(9.6)
" 2.	0.036	29.6	2.4
Feces 1	0.039	38.0	3.3
" 2.	0.025	17.6	1.0
Total			62.2
Rat 3, female, 160 gm.; fed 200 mg. D-tryptophan containing 6.08% N ¹⁵ excess in indole ring			
Urine 1.	0.251	184.8	55.4
" 1, ammonia	0.190	24.4	(5.5)
" 1, urea	0.046	106.2	(5.8)
" 2.	0.040	46.3	2.1
Feces 1	0.082	73.1	7.1
" 2	0.043	11.3	0.7
Total			65.3
Rat 4, male, 177 gm.; fed 140 mg. DL-tryptophan containing 31.2% N ¹⁵ excess in indole ring			
Urine 1.	0.850	162.4	45.1
" 1, ammonia	1.290	15.2	(6.4)
" 1, urea	0.323	108.6	(11.5)
" 2	0.076	54.7	1.4
Feces 1	0.474	7.2	1.1
" 2	0.456	19.4	2.9
Total			50.5

Acetyl-DL-tryptophan isolated from carcass, (Rat 1) 0.013, (Rat 2) 0.170, (Rat 3) 0.251 N¹⁵ per cent excess; m.p. 205-206°.

* The figures in parentheses are excluded from the total.

Following ingestion of D-tryptophan, the finding of excess N^{15} (Rat 3) in the total nitrogen of tryptophan isolated from the carcass is proof of the conversion of D-tryptophan to L-tryptophan in the rat.

Following ingestion of both L- and D-tryptophan, the finding of excess N^{15} (Rats 2 and 3) in the total nitrogen of tryptophan isolated from the carcass demonstrates the incorporation of dietary tryptophan into the body proteins. In both cases it is likely that the N^{15} concentration of the

TABLE III

Amino Acids Isolated from Combined Liver, Spleen, Intestines, and Plasma

Rat 4, fed 140 mg. of DL-tryptophan containing 31.2 per cent N^{15} excess in the indole ring.

Amino acid	Criterion of purity	Values from literature	N^{15}
		degrees	per cent excess
Tyrosine	$[\alpha]_D^{25} = -10.9^\circ$	-10.8	0.022
Leucine	M.p. benzenesulfonylleucine, 128°	129	0.032
Glutamic acid	Glutamic acid hydrochloride, $[\alpha]_D^{25} = +25.5^\circ$	+25.3	0.067
Aspartic acid	Recrystallized 3 times as Cu salt	per cent	0.070
Arginine	Arginine monoflavinate; found N 17.30%	17.21	0.057
Ornithine			0.032
Amidine N			0.079

α -amino group is very low, since studies in this laboratory have demonstrated that tryptophan isolated from rats given N^{15} -glycine contains a lower N^{15} concentration than any other amino acid except lysine.¹

The finding of 0.013 per cent excess N^{15} in the total nitrogen of tryptophan isolated from the carcass of a rat which had received the isotopic indole indicates that there is no significant conversion of indole to tryptophan.

SUMMARY

1. Indole containing N^{15} has been synthesized. DL-Tryptophan containing N^{15} in the indole ring has been synthesized and resolved into the D and L isomers.

2. Studies of the conversion of tryptophan to kynurenine, kynurenic acid, and xanthurenic acid in rats and rabbits are reported. Tryptophan is converted to kynurenine, kynurenic acid, and xanthurenic acid with only slight dilution. D-Tryptophan is utilized by the rat but not by the rabbit.

¹Foster, G. L., unpublished data.

Kynurenine is converted to xanthurenic acid in the rat without dilution, and to kynurenic acid in the rabbit with significant dilution.

3. There is no detectable conversion of the pyrrole ring of tryptophan to the tetrapyrrole structure of hemin in the rat.

4. N^{15} -Labeled indole fed to rats is mainly excreted in the urine. There is very little rupture of the ring and no detectable conversion to tryptophan.

5. D-Tryptophan is readily converted to L-tryptophan, which is incorporated efficiently into the tissue proteins of rats. Both the D and L isomers showed significant rupture of the indole ring. Urinary ammonia had an isotope concentration 4 times as high as that of urinary urea, indicating that the kidney is probably the principal site of the ring-splitting reaction. Fecal excretion of isotope was small.

6. Amino acids isolated from the internal organs of a rat fed a high isotope concentration of DL-tryptophan contained N^{15} , demonstrating that the ring nitrogen of tryptophan can enter the general nitrogen pool.

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QUINOLINE AND ITS TRANSFORMATION PRODUCTS FOUND IN URINE

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Therapeutic agents generally undergo transformation in the body to metabolites that are pharmacologically inactive, or considerably less active than the parent compounds. In relating chemical constitution to therapeutic effect in a series of compounds, it is usually not obvious whether variations in structure decrease or increase inherent pharmacological activity or merely decrease or increase stability in the body.

A number of important therapeutic agents including the cinchona alkaloids, quinine, quinidine, cinchonine, and cinchonidine, possess a quinoline nucleus. These four alkaloids undergo oxidation in the body to form 2-hydroxyquinoline derivatives (1). Knox (2) has isolated from rabbit liver an enzyme which catalyzes *in vitro* the oxidation of the quinoline ring in the 2 position of not only the cinchona alkaloids, but quinoline and certain of its derivatives.

A study was designed to determine the influence of various nuclear substituents on the ease of oxidation of the quinoline ring in the body. It was hoped that knowledge of the relationship between chemical structure and stability *in vivo* might make information available which would permit the synthesis of more stable and possibly more useful therapeutic agents. The first step in this study deals with the metabolism of quinoline in the dog.

The fate of quinoline in the body cannot be deduced from the data and the rather conflicting opinions recorded in the literature. A number of metabolic products have been reported, but in no case does the urinary excretion of a product claimed account for more than a very small fraction of the administered quinoline. Donath (3), in 1881, reported that quinoline was completely metabolized in the human. Although he suggested that the carbocyclic ring was destroyed and a pyridine carboxylic acid thereby formed, no product was isolated from urine to support this hypothesis. Fuhner (4) concluded that 5,6-dihydroxyquinoline appears in the urine of rabbits and dogs receiving quinoline. This conclusion was

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reached on the basis of a color reaction which is said to be characteristic for 5,6-dihydroxyquinoline and was not verified by isolation and identification of the compound. Scheunemann (5) described the isolation of 6-hydroxy-4-quinolone, 8-hydroxyquinoline, and 6-hydroxyquinoline from the urine of rabbits receiving quinoline. Two workers, Tamura (6) and Kamei (7), obtained, from the urine of dogs given quinoline, minor amounts of a compound which they isolated only as a platinum salt. They suggested, on the basis of the platinum and nitrogen content of the platinum salt, that the compound was *N*-methylquinoline.

Chemical Methods

Estimation of Quinoline in Plasma and Urine

Quinoline was extracted from alkalized plasma or urine with heptane, returned to dilute acid, and estimated on the basis of its ultraviolet absorption at 310 m μ . 3-Hydroxyquinoline is not extractable under these conditions. The details of the method are similar to those previously described for the estimation of basic organic compounds by ultraviolet spectrophotometry (8).

Estimation of Total 3-Hydroxyquinoline (Free and Conjugated) in Urine

The conjugated forms of 3-hydroxyquinoline were hydrolyzed to free 3-hydroxyquinoline by acidification of the urine with HCl to about 1 N and then heating on a boiling water bath for 1 hour. The pH was then adjusted to 6 and the 3-hydroxyquinoline extracted with benzene. It was returned to dilute sulfuric acid and its concentration estimated fluorometrically. The details for the procedure are similar to those previously described for the photofluorometric estimation of quinine (9).

The concentration of free 3-hydroxyquinoline was estimated in the urine by adjusting the urine to pH 6 and extracting directly with benzene, the hydrolysis step being omitted.

EXPERIMENTAL

A dog was given 0.5 gm. of quinoline intravenously¹ and urine was collected during the following 24 hours.

Quinoline in Urine—The total urine collected was adjusted to pH 13 with NaOH solution and extracted several times with heptane to remove any quinoline present in the urine. Quinoline-like material extracted by the solvent was then returned to an aqueous phase by shaking with a small volume of dilute HCl. The material (a total of 3 mg. as determined by ultraviolet absorption at 310 m μ) was then subjected to an eight plate counter-current distribution in separatory funnels (10), the solvents being

¹ The quinoline used, c.p. grade, boiled at 235–237°. For intravenous injection the compound was dissolved in a slight excess of 0.1 N hydrochloric acid.

heptane and acetate buffer, pH 4.4. With these solvents the material, as measured by ultraviolet absorption at 310 $m\mu$, was distributed about equally between the two phases. After counter-current distribution, the partition ratio of the material in each separatory funnel was determined by measuring its concentration in each phase. The material in the funnels containing the major portion had almost identical partition ratios, suggesting that only one major component was present. The contents of Funnels 3 to 7 were then pooled. The aqueous phase of the pool was alkalinized with NaOH and the quinoline-like material transferred to the organic

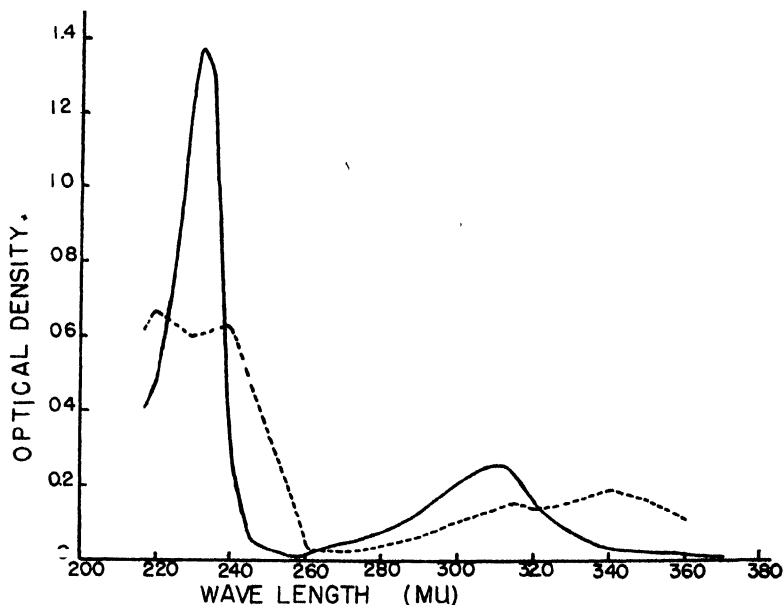


FIG. 1. The absorption spectra of quinoline (solid line) and 8-hydroxyquinoline (dotted line) from urine. The substances were dissolved in 0.1 N HCl at a concentration of 5 γ per cc. Cell thickness, 1 cm.

phase by shaking. It was then returned to 0.1 N HCl. The absorption spectrum of the acid solution was measured and found to be almost identical with that of a known sample of quinoline (Fig. 1). Further evidence that the compound was quinoline was obtained by finding that its partition ratios between heptane and water at various pH values were almost identical with those of authentic quinoline.

Metabolic Transformation Products in Urine—Following the initial heptane extraction (see above under quinoline), the urine was extracted with diethyl ether. The ether phase was then shaken with 0.1 N HCl. The absorption spectrum of the aqueous phase was examined but revealed no

absorption peaks which might suggest the presence of quinoline transformation products.

Following the ether extraction, urine was adjusted to about pH 1 by the addition of concentrated HCl and heated on the steam bath for 1 hour. The urine was adjusted to pH 6 by the addition of solid Na_2CO_3 and then shaken twice with equal volumes of ether. The basic material in the ether phase was then returned to 0.1 N HCl. Measurement of the absorption spectrum revealed considerable amounts of material with an absorption peak at 340 $\text{m}\mu$. A counter-current distribution in the manner described for quinoline, by use of the system diethyl ether-0.75 N acetic acid, yielded only one major component. The contents of Funnels 3 to 7 were pooled. The aqueous phase of the pooled funnels was adjusted to pH 6 and the basic material contained therein was transferred to the ether phase by shaking. The ether was evaporated to dryness and the residue recrystallized from ethylene dichloride. The yellowish crystals which resulted were dissolved in hot 10 per cent ethanol-water and decolorized with activated charcoal. Cooling of the filtered solution yielded white needles melting at 198° (uncorrected). Further recrystallization did not change the melting point. The compound was sparingly soluble in water but dissolved readily in both acid and alkaline solutions. It exhibited fluorescence in dilute sulfuric acid. The compound gave the following per cent composition on analysis.

$\text{C}_9\text{H}_7\text{NO}$. Calculated.	C 74.48, H 4.83, N 9.66
Found.	" 74.68, " 4.70, " 9.56

The analysis of the compound indicates that it contains 1 more oxygen atom than does quinoline. This together with the amphoteric properties of the metabolite, suggests that it is one of seven possible hydroxyquinolines. 3-Hydroxyquinoline has a recorded melting point of 198° (uncorrected) (11). The *p*-toluenesulfonyl derivative of the metabolite was prepared and was found to melt at 88° (uncorrected), agreeing closely with the 90° recorded for the 3-hydroxyquinoline derivative (12).

3-Hydroxyquinoline was synthesized by a method described by Mills and Watson (11). The synthetic compound melted at 198° (uncorrected) and a mixture with the quinoline metabolite showed no depression of the melting point. The *p*-toluenesulfonyl derivative of the synthetic material was prepared and melted at 88° (uncorrected). A mixture of the *p*-toluenesulfonyl derivatives of the synthetic compound and the metabolite exhibited no depression of the melting point. The absorption spectra of the apparent 3-hydroxyquinoline and the synthetic compound in 0.1 N H_2SO_4 were almost identical (Fig. 1) and agree with the spectrum recorded for 3-hydroxyquinoline (13). Both substances were found to exhibit fluo-

rescence in acid solution. This fluorescence was quenched by chloride ions and exhibited similar changes with variations in pH.

The amounts of quinoline and 3-hydroxyquinoline excreted in urine were measured subsequent to the administration of quinoline. Three dogs were given quinoline by intravenous injection. Two animals received 20 mg. per kilo and the third 25 mg. per kilo. Urine was collected for 24 hours after administration of the substance. The excretion of quinoline or its metabolite was negligible subsequent to this time. In each case, less than 0.5 per cent of the administered quinoline was excreted unchanged, indicating that the drug is almost completely metabolized in the body. 3-Hydroxyquinoline, equivalent to 29, 31, and 32 per cent of the administered quinoline, was recovered from the urine of the three dogs. An average of 4 per cent appeared as the free hydroxyquinoline and the remainder as a conjugated form, possibly the glucuronate or sulfate or both.

TABLE I

Plasma Quinoline Following Intravenous Administration of 25 Mg. Per Kilo of Quinoline to Dog

Time	Concentration of quinoline in plasma
hr.	mg. per l.
$\frac{1}{2}$	16.9
$\frac{3}{4}$	5.1
2	2.6
4	0.7

The rate of metabolic transformation of quinoline was studied in the dog. 25 mg. per kilo were administered intravenously and plasma quinoline concentrations observed during the next 4 hour interval (Table I). It is apparent from the data that the drug is metabolized rapidly, since at 4 hours there is little quinoline remaining in the plasma.

The fate of 3-hydroxyquinoline was then studied. The compound was administered intravenously in dilute HCl to two dogs in amounts of 0.6 mg. per kilo. Urine was collected over a period of 24 hours. 34 per cent and 35 per cent of the administered dose of 3-hydroxyquinoline were recovered in the urine in a conjugated form. Free 3-hydroxyquinoline was found in only negligible amounts. The remainder of the compound was metabolized through an unknown route.

DISCUSSION

In the dog, about one-third of an administered dose of quinoline is excreted as 3-hydroxyquinoline, mainly in a conjugated form. It is to be

noted that approximately the same fraction of 3-hydroxyquinoline is excreted after the direct administration of the latter compound to the dog. This suggests that quinoline is almost entirely routed through an initial oxidation to 3-hydroxyquinoline. This view must be accepted with some reservation, however, since the doses of 3-hydroxyquinoline administered were considerably smaller than the comparable doses of quinoline. Nevertheless, the fact that administered 3-hydroxyquinoline can be only partly accounted for in the urine shows that further metabolic transformation of this compound takes place.

The formation of only 3-hydroxyquinoline in detectable amounts is unexpected when it is considered that quinoline derivatives such as the cinchona alkaloids are oxidized in man not in the 3 position but in the 2 position of the quinoline ring (1). Furthermore, Knox (2) showed the presence in rabbit liver of an enzyme which catalyzes *in vitro* the oxidation of quinoline, as well as its derivatives, in the 2 position. That a similar enzyme operates in the dog *in vivo* is suggested by the conversion of cinchonine to 2-hydroxycinchonine in this animal also.² The possibility exists, of course, that a part of the quinoline is oxidized in the 2 position and that the resulting compound is completely metabolized to non-identifiable material.

SUMMARY

Quinoline is almost completely metabolized in the dog. The main route of metabolism appears to be an initial oxidation to 3-hydroxyquinoline, a substance which is found in the urine mainly in a conjugated form.

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MAMMALIAN TYROSINASE: THE RELATIONSHIP OF COPPER TO ENZYMATIC ACTIVITY

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Copper has been reported to be an essential part of the enzyme tyrosinase prepared from various plant and insect sources (1-8). In the case of potato tyrosinase, Kubowitz (1, 2) found that the enzyme could be inhibited by reagents which combine with copper, *e.g.* diethyldithiocarbamate, salicylaldehyde, and carbon monoxide. He demonstrated that treatment of the enzyme with cyanide followed by dialysis resulted in a decrease in the copper content of the enzyme preparation and a loss of enzymatic activity. Addition of sufficient cupric ions resulted in practically complete restoration of activity. Other metals (iron, cobalt, nickel, manganese, and zinc) were ineffective in restoring enzymatic activity. Allen and Bodine (3) carried out experiments on protyrosinase from grasshopper eggs similar to those of Kubowitz and obtained essentially the same results.

Several investigators have shown that copper is associated with the activity of phenoloxidases from mushrooms and tea leaf (4-8).

Hogeboom and Adams (9) demonstrated an inhibition by cyanide of the enzymatic oxidation of L-tyrosine and dihydroxyphenyl-L-alanine (dopa), using extracts from the Harding-Passey mouse melanoma; they concluded that the enzymes concerned in the oxidation of tyrosine and dopa by melanotic tissue are iron- or copper-containing enzymes. Cunningham (10) and Greenstein *et al.* (11) found that copper is present in human melanomas.

The experiments reported here present further evidence that mammalian tyrosinase prepared from the Harding-Passey mouse melanoma is an enzyme which requires copper for activity. This evidence is based on the demonstration that copper can be removed from the enzyme preparation with cyanide to produce a relatively inactive enzyme and that the addition of copper to such a preparation restores activity. Furthermore, sub-

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stances that combine with copper inactivate the enzyme and this inactivation is, in some instances, reversed by the addition of excess copper.

EXPERIMENTAL

Tyrosinase was prepared from the Harding-Passey mouse melanoma by the following procedure: Fresh mouse melanoma tissue was ground with sand and cold distilled water for 10 minutes. The mixture was treated in a Waring blender at 5° for 10 minutes and then centrifuged at 3000 r.p.m. for 30 minutes. The supernatant fluid was dialyzed twice against 100 times its volume of cold distilled water for 48 hours. The final solution had approximately 4 times the volume of the original tumor tissue. Enzymatic activity was determined manometrically by measurements of the oxygen uptake in the Warburg apparatus at 38°, in 0.1 M potassium phosphate buffer at pH 6.8. The substrate,¹ 0.5 mg. of dopa in 0.1 M potassium phosphate buffer, was added from the side arms of the Warburg vessels to the enzyme preparation after 10 minutes equilibration of the solution at 38°. Autoxidation of dopa under these conditions is negligible for the time intervals of the experiments. In general, 3 units of enzyme were used per vessel which contained 3 ml. of reaction mixture. 1 enzyme unit is defined (12) as the amount of enzyme required to catalyze the absorption of 1 μ l. of oxygen per minute by 1 mg. of substrate (dopa) when oxidation is proceeding at a maximal rate.

Removal of Copper with Cyanide—A method of removing copper from tyrosinase similar to that used by Kubowitz (1, 2) and Allen and Bodine (3) was followed. The enzyme preparation used in this experiment was made according to the method given above, except that the preparation was not dialyzed until after treatment with potassium cyanide. All of the procedures which follow were carried out at 5°. To 15 ml. of enzyme preparation were added 10 ml. of 1 M KCN solution and the mixture was allowed to stand overnight. The enzyme was precipitated by adding 50 ml. of saturated ammonium sulfate solution, which was likewise saturated with potassium cyanide. After 2 hours, the mixture was centrifuged at 3000 r.p.m. for 1 hour. The supernatant fluid was discarded, 10 ml. of distilled water were added to the precipitate, and the mixture was dialyzed in a cellophane bag against 2000 ml. of cold distilled water. After 4 hours, a change of water was made and dialysis was further carried out for 15 hours. The water was changed again and the dialysis was continued for another

¹ Dopa was ordinarily used as the substrate in the present study because changes in enzymatic activity could be readily observed. The enzymatic oxidation of tyrosine is complicated by the induction period in the conversion of tyrosine to dopa (12). As indicated in the text, however, tyrosine was used as the substrate in a few of the experiments.

24 hours. The dialyzed preparation was then diluted to a volume of 26 ml. with distilled water. A similar volume of tyrosinase preparation used as a control received identical treatment, except that distilled water was used in place of potassium cyanide solutions, and saturated ammonium sulfate without potassium cyanide was used to precipitate the control tyrosinase.

The quantitative copper determinations on the enzyme preparations were carried out by drying the samples, ashing at 450° , and subsequent colorimetric estimation of copper in the ash solutions with diethyldithiocarbamate. The copper content of the enzyme preparation was 0.19 per cent. After treatment with cyanide, the enzyme preparation contained 0.09 per cent copper.

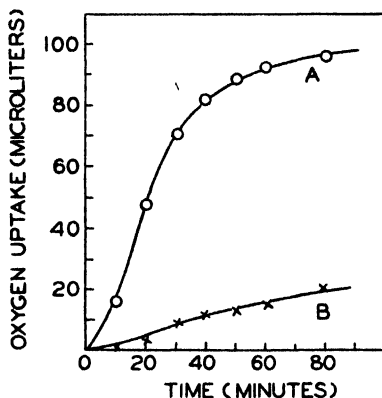


FIG. 1. The effect of previous treatment with cyanide on the ability of mouse melanoma extract to catalyze the aerobic oxidation of dopa (0.5 mg.) at pH 6.8; 38° . Curve A, untreated extract; Curve B, after treatment with cyanide.

The activities of the tyrosinase preparations before and after treatment with cyanide, with dopa as the substrate, are demonstrated in Fig. 1. It can be seen that approximately 85 per cent of the enzymatic activity was lost after most of the copper had been removed.

The addition of cupric ions (as copper sulfate) to the cyanide-treated enzyme preparation resulted in restoration of enzymatic activity, the extent of restoration being a function of the concentration of added cupric ions; with sufficient added copper, complete enzymatic activity was restored (Fig. 2). Relative per cent restoration of activity was determined from the ratios of the maximal amount of oxygen absorbed during a 10 minute interval by the cyanide-treated tyrosinase (with varying amounts of added cupric ions) to the oxygen absorbed by the control tyrosinase.

Addition of the amount of copper that was removed by cyanide treat-

ment resulted in a restoration of approximately 90 per cent of the activity of the control tyrosinase. Kubowitz (1, 2) found it necessary to add about 10 times the amount of copper originally present to his copper-free potato tyrosinase to obtain complete restoration of activity. Allen and Bodine (3) found that twice as much copper as was originally present in grasshopper egg protyrosinase was necessary to restore complete activity.

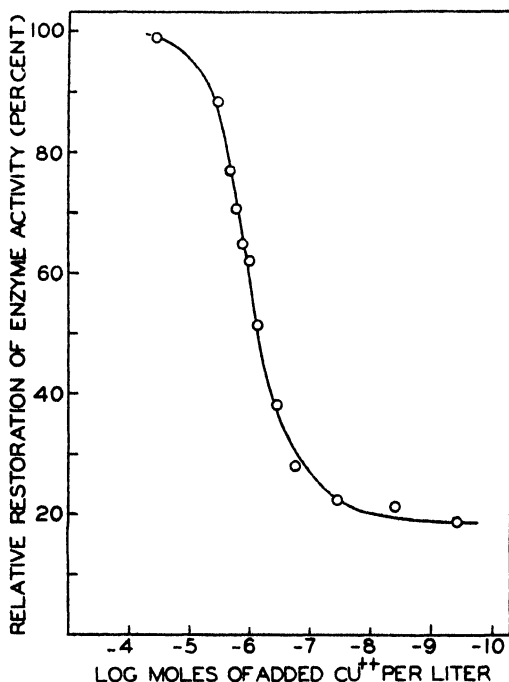


Fig. 2. The effect of added Cu^{++} , at a constant enzyme concentration, on the restoration of enzymatic activity to a cyanide-treated dialyzed tyrosinase preparation from mouse melanoma. Substrate, dopa (8.45×10^{-4} molar); pH 6.8; 38° .

When 0.5 mg. of tyrosine was used as the substrate in place of 0.5 mg. of dopa, there was no oxygen uptake with the cyanide-treated tyrosinase, although the control preparation catalyzed the oxidation of tyrosine after an induction period of approximately 60 minutes. Furthermore, in the presence of sufficient cupric ions to restore 100 per cent activity against dopa as the substrate, there was likewise no oxidation of tyrosine even after 4 hours. However, when small amounts of dopa (0.05 mg.) were added to this copper-activated tyrosine-tyrosinase mixture, there was complete restoration of tyrosinase activity and the rate of tyrosine oxidation was equal to that obtained with the control tyrosinase under similar conditions.

In addition to copper sulfate, the following solutions of metallic salts were added to the cyanide-treated tyrosinase: ferric nitrate, zinc sulfate, cobaltous sulfate, magnesium chloride, manganese chloride, and nickelous sulfate. These substances were added in amounts equivalent to 10 times the amount of copper sulfate required to restore 100 per cent activity. Some enzymatic activity was restored with nickelous sulfate, but none of the other metals had any effect.

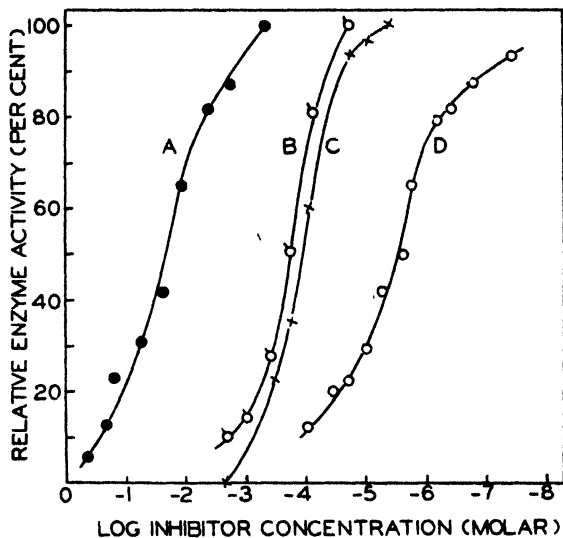


Fig. 3. The effect of varying concentrations of inhibitors on the enzymatic activity, at constant enzyme concentration, of mouse melanoma extracts in the oxidation of dopa (8.45×10^{-4} molar) at pH 6.8; 38° . Curve A, thiourea; Curve B, 2,3-dithiopropanol (BAL); Curve C, diethyldithiocarbamate, Curve D, phenylthiourea.

In addition to potassium cyanide as an inactivator, several sulfur-containing organic compounds which form complexes with heavy metals were studied. The compounds studied were thiourea, thiouracil, glutathione, cysteine, 2,3-dithiopropanol (BAL), diethyldithiocarbamate, and phenylthiourea. Compounds of these types inhibit various plant tyrosinases (1, 13-16).

Varying amounts of each of these compounds were allowed to incubate with constant amounts of enzyme (approximately 3 units) in the main reaction compartment of the Warburg vessels at 38° for 30 minutes. After this time 0.5 mg. of dopa was added from the side arms, and the oxygen consumption was recorded at 10 minute intervals for 120 minutes. The per cent activity of the tyrosinase treated with a given compound relative to that of the control tyrosinase was calculated from the ratios of the maxi-

mal values of the oxygen uptake during a 10 minute interval for the respective preparations. The results for four of these compounds are shown in Fig. 3. The inhibition curves (not shown) for glutathione, cysteine, and thiouracil had the same general S-shaped form as those shown in Fig. 3, falling between Curves A and B. The negative logarithm of the inhibitor concentration producing 50 per cent inhibition by the various inhibitors studied is recorded in Table I.

Reversal of Tyrosinase Inhibition by Addition of Copper—To study the possible reversal of tyrosinase inhibition by the addition of copper, concentrations of inhibitors giving approximately 90 per cent loss of activity were incubated with the enzyme in the Warburg apparatus as described above. After adding the substrate, dopa, the reaction was allowed to run for 40 minutes. At this time, cupric sulfate in 25 per cent excess of the inhibitor

TABLE I

Relative Effectiveness of Various Inhibitors against Mammalian Tyrosinase at pH 6.8; 38°

Inhibitor (<i>I</i>)	— Log <i>I</i> (molar) for 50 per cent inhibition
Thiourea.. . . .	1.75
Thiouracil.....	2.05
Glutathione .. .	2.35
Cysteine	2.90
2,3-Dithiopropanol (BAL)	3.85
Diethyldithiocarbamate	4.00
Phenylthiourea...	5.60

on a mole for mole basis was added from a second side arm to the reaction mixture. The inhibition of the reaction in the cases of diethyldithiocarbamate and BAL was reversed by the added cupric ions. An example of such reversal of inhibition by copper is given in Fig. 4. The inhibition produced by phenylthiourea could not be reversed by as much as 100 per cent excess of copper. Reversal by copper was not studied for thiourea, thiouracil, glutathione, and cysteine because addition to the reaction mixture of the amount of copper sulfate necessary to give an excess over the inhibitor resulted in a precipitate with the phosphate buffer.

When tyrosine was used as the substrate instead of dopa, an effect similar to that obtained in the case of cyanide inhibition was observed; *viz.*, the addition of an excess of cupric ions to tyrosinase inhibited by diethyldithiocarbamate or BAL did not restore activity toward tyrosine unless a small amount of dopa was also present. That is, an excess of cupric ions restored activity of a diethyldithiocarbamate or BAL-inhibited tyrosinase

toward dopa but the preparation then had an indefinitely prolonged induction period in the oxidation of tyrosine.

The metal salts, ferric nitrate, zinc sulfate, cobaltous sulfate, magnesium chloride, and manganese chloride, had no effect on reversing the inhibition produced by diethyldithiocarbamate and BAL. Nickelous sulfate did not reverse diethyldithiocarbamate inhibition but did restore slight activity to the BAL-inhibited tyrosinase.

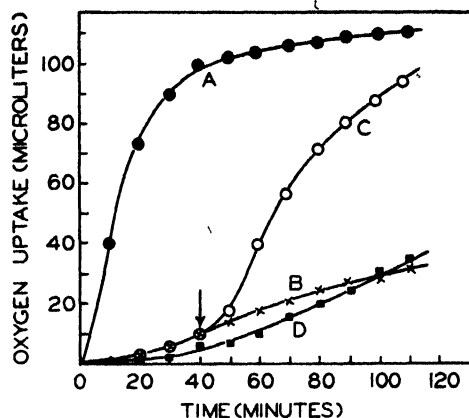


FIG. 4. The inhibitory effect of diethyldithiocarbamate on the enzymatic oxidation of dopa by mouse melanoma preparation and the reversal of inhibition by Cu^{++} ; pH 6.8; 38° . Curve A, enzyme + dopa; B, enzyme + dopa + diethyldithiocarbamate; Curve C, the same as Curve B but with Cu^{++} added at the time indicated by the arrow; Curve D, dopa + Cu^{++} , no enzyme.

DISCUSSION

The experimental evidence reported above indicates that tyrosinase from the Harding-Passey mouse melanoma is an enzyme which requires copper for its activity. When the enzyme preparation is treated with potassium cyanide and dialyzed, much of the copper originally present in the preparation is removed. The "copper-free" tyrosinase has little enzymatic activity. The addition to this preparation of an amount of cupric ions equal to the amount of copper removed by the cyanide-dialysis treatment restored approximately 90 per cent of the original activity. Ferric, zinc, cobaltous, magnesium, and manganese ions did not produce any effect. Nickelous ions restored some activity but were not as effective as cupric ions.

The enzyme is inhibited by certain organic thio compounds. It is likely that these compounds exert most of their action by combining with the copper required for enzymatic activity. The inhibition of tyrosinase by

the thio compounds can in some instances be reversed by the addition of an excess of cupric ions. Other metal ions do not affect the inhibition.

It is interesting to note that, when tyrosine is used as the substrate for tyrosinase inhibited by cyanide treatment or by the compounds listed in Table I, activity is not restored by the addition of cupric ions unless a small amount of dopa is also present. That is, "reactivated" tyrosinase has an indefinitely prolonged induction period in the oxidation of tyrosine alone, yet in the presence of small amounts of dopa the reactivated enzyme is fully as active against tyrosine as was the original untreated enzyme. This parallels our earlier experience with various fractionation procedures supposedly designed to separate "tyrosinase" from "dopa oxidase," and is further evidence that under certain circumstances the distinction between these two activities is no longer valid (12).

It is an extremely difficult task to prove categorically that an enzyme molecule itself contains copper. For such proof, one would have to obtain a pure enzyme preparation that satisfies all the criteria required of a homogeneous preparation. One would then have to demonstrate the constancy of the copper content in the active enzyme under a variety of conditions. Finally, removal of copper should lead to a loss of enzymatic activity. Activity should be restorable on the addition of sufficient copper in the proper form.

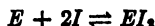
We have not been able to carry out this detailed proof to show that mammalian tyrosinase is a copper-containing enzyme because we have not as yet been able to obtain a pure tyrosinase preparation. Mammalian tyrosinase activity is associated with the particulate matter of cells (12) and no way has been found to free the enzyme from the particles or to obtain particulate preparations with purely tyrosinase activity. Even without this rigorous proof, however, we feel that the evidence presented above justifies the conclusion that copper is as firmly associated with mammalian tyrosinase activity as it is with tyrosinase from plant and insect sources.

The inhibition curves shown in Fig. 3 vary somewhat in shape. The curves for BAL and diethyldithiocarbamate inhibition have a steep slope and correspond in form to the theoretical curve resulting from plotting the enzymatic activity against the logarithm of the total inhibitor concentration in accordance with the following equation:

$$- \left(\frac{1 - a}{a} \right) K + 2(1 - a)E \quad (1)$$

where I = the molar concentration of the inhibitor (total), E = the molar concentration of the enzyme (total), a = the fractional activity of the enzyme, and K = the dissociation constant of the enzyme-inhibitor complex.

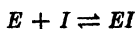
This equation represents the following enzyme-inhibitor reaction (17):



The curve for thiourea inhibition is similar to the theoretical curve obtained from the relationship:

$$I = \left(\frac{1-a}{a} \right) K + (1-a)E \quad (2)$$

This relationship corresponds to the following enzyme-inhibitor reaction:



The shape of the activity-inhibitor curves (Fig. 3) may indicate the number of molecules of inhibitor which combine with active enzyme centers. Thus one might conclude from our data that either 1 or 2 molecules of thiourea, BAL, or diethyldithiocarbamate combine with one active enzyme center. However, the experimental difficulties associated with obtaining precise curves, together with the admitted complexity of the enzyme system concerned, make us hesitant to reach such definite conclusions concerning the stoichiometric relation between enzyme and inhibitor until more adequate data are available. In this connection, it is interesting to note that DuBois and Erway (13), using potato tyrosinase and inhibitors similar to those described here, found straight line relationships between per cent activity and the logarithm of inhibitor concentrations. However, their data cover the range of activity between 20 and 85 per cent only, and in this range it is difficult to establish the true shape of the activity-inhibitor curve.

The phenylthiourea inhibition curve cannot be represented by any simple enzyme-inhibitor reaction. The fact that this inhibition cannot be reversed by cupric ions indicates that phenylthiourea may inhibit tyrosinase by a way other than that of binding copper.

The curve in Fig. 2, which represents the restoration of enzymatic activity by the addition of cupric ions to a "copper-free" tyrosinase preparation, is of somewhat the same type as the activity-inhibitor curves just discussed, but is even less susceptible to quantitative interpretation. This is no doubt due in part to our inability to obtain an enzyme preparation which was completely free from copper and also devoid of enzymatic activity. However, the relation between added copper and enzyme activity shown in Fig. 2 suggests to us the strong possibility that it represents a reversible equilibrium between enzyme molecules and copper ions. Further study will likewise be necessary to clarify this point.

SUMMARY

1. Mammalian tyrosinase requires copper for enzymatic activity.
2. Compounds that combine with copper inhibit mammalian tyrosinase; in some instances, this inhibition can be reversed by added cupric ions. Other metal ions do not have this effect.
3. The nature of the reaction between tyrosinase and inhibitors and between "copper-free" tyrosinase and copper is discussed.

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THE KINETICS OF TRYPSIN DIGESTION*

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The present study of a typical proteolytic enzyme extends the quantitative investigations previously reported from this laboratory on the effects of pressure on protein systems *in vivo* (1) and *in vitro* (2). This paper presents new data on the self-digestion of trypsin and the digestion of casein by trypsin; a forthcoming paper will discuss the effects of temperature and hydrostatic pressure on these processes. This particular system was chosen because of classical interest, because of the commercial availability of crystalline trypsin and purified casein, and also because some investigations of the kinetics of digestion of casein by trypsin are already to be found in the literature (3).

As is frequently the case, the rate law suggested by the interpretation of the data has a complex integrated form not easily tested by the usual methods. A simple graphical technique (which we have called the slide-fit technique) has proved useful in handling kinetic data in general (Powell, unpublished data). With this technique it is quite as easy to test the agreement of data with complex rate laws as with the simple classical rate laws.

Section I of this paper presents an analysis of trypsin self-digestion data. The rate of this process was studied to find out whether it might proceed rapidly enough to introduce any significant error into the studies of casein digestion. The data, while not intended for kinetic analysis, are presented as a simple illustration of the slide-fit technique and because the kinetic process, second order digestion complicated by reversible inhibition of the enzyme by its digestion products, appears completely parallel to the casein digestion.

Section II presents new data covering a wide range of the essential variables of casein-trypsin digestion and interprets them similarly in terms of known properties of the components.

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I. Slide-Fit Technique and Trypsin Self-Digestion

Experimental—Conditions in the trypsin experiments were exactly like those for casein-trypsin (Section II), except that samples were taken from a single stock. A freshly made solution of 3 mg. per ml. of crystalline trypsin (Worthington Laboratory, assay 50 per cent) in very dilute hydrochloric acid (pH 1.96) was mixed in the proportion 1.1 to 1.5 with 0.1 M phosphate buffer of pH 7.3, and the mixture (pH 7.1, chosen to duplicate the casein conditions which, in turn, approximate Kunitz' conditions) was stirred mechanically at 35°. Part (2.6 ml.) of each sample was precipitated with 5 per cent trichloroacetic acid (TCA) and the optical density of the filtrate determined at 280 m μ , and part (1.1 ml.) was allowed to digest 1.5 ml. of 1 per cent casein in the same buffer under standard condi-

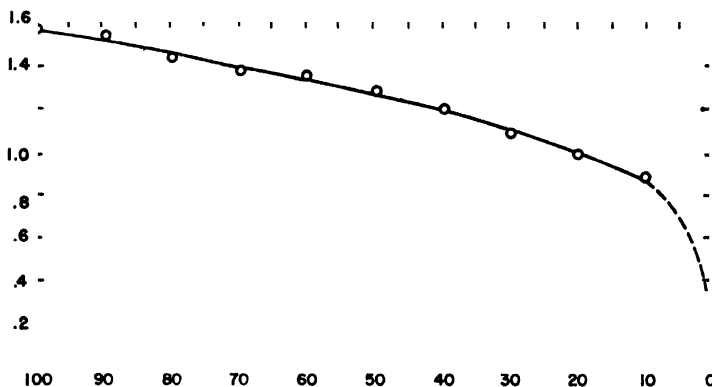


FIG. 1. Activity of trypsin measured by casein digested in 20 minutes under standard conditions. Ordinate, optical density; abscissa, amount of trypsin charged as per cent of normal charge (see the text).

tions (35°, 20.0 minutes) as a measure of its residual enzymatic activity. The amount of casein digested was determined by measuring the optical density of a TCA filtrate at 280 m μ , and correcting for the optical density of the first part of the sample, *i.e.*, the contribution of self-digested trypsin (Table I).

Calibration—A similar 3 mg. per ml. solution of trypsin was diluted with varying amounts of the dilute hydrochloric acid so that successive samples contained amounts differing by 10 per cent increments. Each sample was then diluted (1.1 ml. + 1.5 ml.) with the phosphate buffer (to reproduce the self-digestion conditions above) and 1.1 ml. of this solution was added to 1.5 ml. of 1 per cent casein and incubated; the optical density of a TCA filtrate was determined. The resulting per cent of active trypsin *versus* the optical density of the casein digested is shown in Fig. 1.

The data for Sample 3-T₀ (Table I) were then converted to per cent of

TABLE I
Trypsin Self-Digestion

Time, min.	Self-digest, optical density	Casein digest, optical density	Corrected* optical density	Per cent undigested
Sample 3-T ₀ (initial concentration, 1.27 mg. per ml. of 50 % trypsin)				
3	0.173	1.500	1.455	80†
6	0.195	1.466	1.412	72
10	0.222	1.478	1.412	72
15	0.250	1.398	1.321	55‡
20	0.270	1.548	1.462	82‡
30	0.294	1.434	1.338	58
40	0.325	1.453	1.344	59
60	0.346	1.381	1.263	47
90	0.385	1.349	1.214	42
120	0.395	1.298	1.159	35
Sample 1-T ₀ (initial concentration ½ of that above)				
1	0.096			84§
2	0.148			55‡
4	0.101			80
5	0.098			82
7	0.102			80
10	0.104			78
19	0.123			68
35	0.137			60
60	0.150			53
90	0.167			43

* Correction = 11/26 (optical density of self-digest - 0.067) where 0.067 is the blank.

† The figures in this column are from Fig. 1.

‡ Points omitted in Fig. 3.

§ The figures in this column are from Fig. 2.

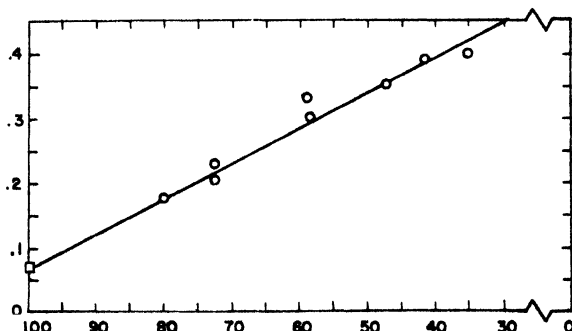
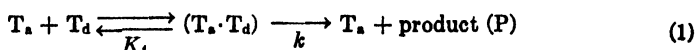


FIG. 2. Trypsin calibration. Ordinate, optical density at 280 mμ from partially self-digested trypsin; abscissa, per cent active or undigested trypsin as determined from activity in digesting casein under standard conditions. □, blank experiment.

active trypsin by use of Fig. 1, and these values, as determined by casein digestion ability, are plotted against the optical density (Table I) of partially digested trypsin in Fig. 2. This is a direct calibration which can be used (as with the data for Sample 1-T₀) to determine the amount of digested trypsin in a solution without recourse to the cumbersome procedure of casein digestion.

DISCUSSION AND RESULTS

Simple Orders of Reaction—Trypsin is known to exist in dynamic equilibrium with a reversibly denatured form of itself (4); it is presumably this form of the protein which is digested by the active enzyme. Thus¹



Since the denaturation equilibrium (4) and complex formation equilibrium (theory of absolute reaction rates) are attained very rapidly, their constants may be combined with the specific rate constant, and the trypsin considered to be a single reacting species, T, giving the rate equation

$$\frac{dP}{dt} = -\frac{dT}{dt} = k'(T_a)(T) = k(T)^2 \quad (2)$$

If α is the degree of reaction and T₀ the original charge of trypsin, then

$$P = \alpha T_0 \text{ and } T = (1 - \alpha) T_0$$

Substituting, rearranging, and integrating formally give

$$\int_0^\alpha \frac{d\alpha}{(1 - \alpha)^2} = T_0 k \int_0^t dt$$

We may combine the constants and take the logarithm of both sides

$$\log \int_0^\alpha \frac{d\alpha}{(1 - \alpha)^2} = \log k' + \log t$$

After integrating, we may then choose a purely arbitrary set of values for α , covering any range desired, and obtain a corresponding set of values for the log of the integral *without recourse to any data whatsoever*. This is a purely mathematical evaluation of the general second order reaction for given values of α . (Derivations for other orders are made similarly.)

¹ Throughout the paper the following terminology is used. T₀ and C₀ refer to initial concentrations; a 2C-T experiment is one with 2 times the "standard" casein concentration (see "Experimental"); C-xT refers to a set of experiments with varying T₀; T_a is active trypsin; T_d is reversibly denatured trypsin; T_a·T_d, etc., are the intermediate complexes; K_i, K_s, K_p are equilibrium constants and the k's are rate constants of the digestion reactions; P is unspecified reaction product; C is unreacted casein; C_d is digested casein.

A plot of α against these values of its integral is, from the above equation, the equivalent of a plot of α against $\log k' + \log t$. Since $\log k'$ is a constant, it is obvious that $\log t$ must describe the shape of the curve and $\log k'$ only its position.

We may now take any data presumed to follow the above rate law and plot the *obtained* values of α against $\log t$. If the reaction mechanism is not correct, the plot of α against $\log t$ will give, in general, a curve of different shape which cannot be superimposed on the hypothetical curve by a shift of the abscissa. If they do fit, the position along the abscissa determines k' .

Comparison of Data to Simple Orders of Reaction—A set of theoretical curves for zero through the third order for the slide-fit is shown in Fig. 3

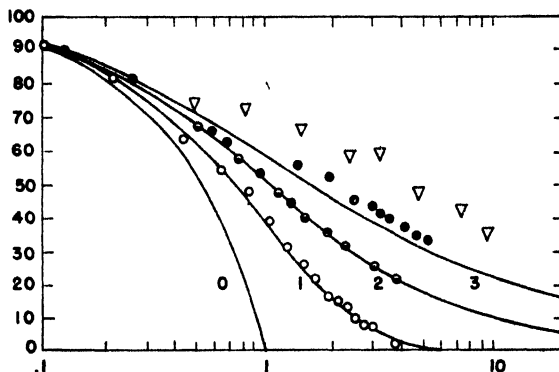


FIG. 3. Theoretical curves for zero, first, second, and third orders of reaction showing fit of data of known first (○) and second (◐, ◑) order reactions, trypsin self-digestion (▽) and trypsin-digested casein (●). Ordinate, per cent unreacted; abscissa, theoretical rate integral, logarithmic scale (see the text).

with the per cent of undigested trypsin (rather than α) plotted against the log of the integral. The data are fitted by plotting the theoretical curve and the data on identical paper and shifting the two sheets horizontally, always keeping the x axis superimposed. The data are then traced in the position of best fit and the reaction k is determined by noting the value of the theoretical curve for unit time of the data. To illustrate (Fig. 3), the data of Lauffer and Price (5) for the demonstrated first order (5, 2) thermal denaturation of tobacco mosaic virus and the data of Hughes and Ingold (6) for the second order decomposition of phenethyl ammonium hydroxide in water have been used. In contrast, the data for trypsin self-digestion (and casein data) are seen to show a rate drop even faster than third order.

Inhibition by Reaction Products—Returning to the original simple reaction mechanism (Equations 1 and 2), we may write the expression for the

KINETICS OF TRYPSIN DIGESTION

activated complex equilibrium

$$K_t = \frac{(T_a \cdot T)}{(T_a)(T)} \text{ or } K_t(T) = \frac{(T_a \cdot T)}{(T_a)} \quad (3)$$

If we now assume that the active trypsin is inhibited by the products in a simple reversible manner (*cf.* (7)), the following equations result:

$$T_a \cdot P, \quad K_p = \frac{(T_a \cdot P)}{(T_a)(P)} \quad \text{or} \quad K_p(P) = \frac{(T_a \cdot P)}{(T_a)} \quad (4)$$

The presumption that the inhibition is reversible is likely from the corresponding reaction of the casein-trypsin system which is discussed below. The total amount of undigested trypsin present is

$$(T) = (T_a) + (T_a \cdot T) + (T_a \cdot P)$$

If we now add the above equilibrium expressions, Equations 3 and 4, and add 1 to each side of the resulting equation

$$1 + K_t(T) + K_p(P) = \frac{(T_a)}{(T_a)} + \frac{(T_a \cdot T)}{(T_a)} + \frac{(T_a \cdot P)}{(T_a)}$$

or

$$(T_a) \frac{(T_a) + (T_a \cdot T) + (T_a \cdot P)}{1 + K_t(T) + K_p(P)} = (T) \frac{(T)}{1 + K_t(T) + K_p(P)}$$

The rate equation

$$-\frac{dT}{dt} = k(T_a)(T)$$

may now be rewritten by substituting for T_a

$$-\frac{dT}{dt} = k \frac{(T)^2}{1 + K_t(T) + K_p(P)}$$

Actually in both this and the casein-trypsin system comparison of the alternatives with the data has shown that the amount of T_a not combined with the product is arithmetically negligible compared to that which is in the form $(T_a \cdot P)$; *i.e.*, $1 + K_t(T) \ll K_p(P)$. Although the development may be carried through with the above form, it is simpler to introduce this fact and to reintroduce the variable α for the degree of reaction. Then

$$T_0 \frac{d\alpha}{dT} = k \frac{(1 - \alpha)^2(T_0)^2}{K_p(\alpha T_0)}$$

and after rearrangement and integration

$$\frac{\alpha}{1 - \alpha} - \ln \frac{1}{1 - \alpha} = \frac{k}{K_p} t \quad (5)$$

This single parameter expression for the self-inactivation or self-digestion of trypsin would obviously be difficult to test by ordinary methods, since it cannot be solved simply for α . By the slide-fit technique, however, it may be treated exactly the same as the above examples. Arbitrary values for α are chosen, substituted, and plotted against the corresponding evaluation of the integral, as before, on semilogarithmic paper. This leads to the curve of Fig. 4.

It can be seen from Equation 5 that if the hypothesis is correct the rate of self-digestion should be independent of the starting concentration. When the results of experiments at two different concentrations are plotted on the same type of paper with the per cent which has reacted (or not reacted, actually) against time and the points fitted to the above theo-

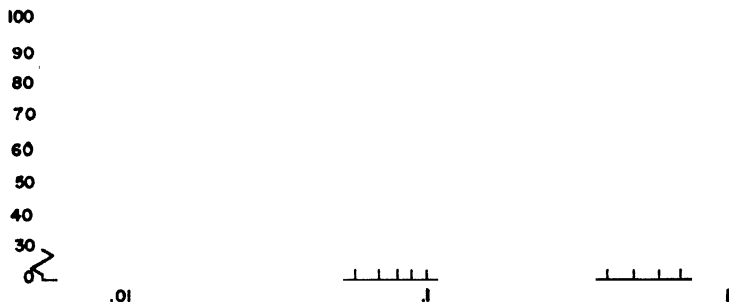


FIG. 4. Trypsin self-digestion data at two concentrations fitted to a theoretical curve describing first order inhibition of the trypsin by the products of reaction. Ordinate, per cent undigested; abscissa, theoretical rate integral, logarithmic scale (see the text). ●, normal concentration ($1-T_0$). ○, triple normal concentration ($3-T_0$).

retical curve, the fit is striking, as may be seen in Fig. 4. The rate constant (value of the theoretical curve for time = 1 minute) is 0.0052 (off scale as drawn).

Other Hypotheses—In addition to the two factors deleted in the derivation of Equation 5, various other theories were considered. The hypotheses and unfavorable results were parallel to those described for casein-trypsin digestion in Section II.

II. Digestion of Casein by Trypsin

Experimental—The standard experiment comprises the digestion at 35.0° of 1 per cent Difco "isoelectric" casein by approximately two-thirds its volume of 1 mg. per ml. of crystalline trypsin at pH 7.10 (chosen to parallel Kunitz' and Northrop's conditions). This is referred to as a C-T experiment. An experiment with twice as much initial casein is a 2C-T or with half as much trypsin a C-½T experiment.

The casein is prepared by shaking 1.000 gm. of Difco isoelectric casein into 75 to 80 ml. of 0.1 M disodium hydrogen phosphate, stirred mechanically as rapidly as possible, *without engendering foaming*. After adjustment to pH 7.30 at 25° with 0.1 M potassium dihydrogen phosphate (about 12 to 13 ml.), the solution is made up to 100 ml. in a volumetric flask with 0.1 M disodium hydrogen and potassium dihydrogen phosphates mixed to pH 7.30 at 25°. This solution will keep 3 to 4 days in the refrigerator.

The trypsin solution used is 1 mg. per ml. (Worthington Laboratory) "once purified crystalline trypsin" (assay 50 per cent) in refrigerated hydrochloric acid of pH 1.96. The solution must be kept iced and should be used immediately.

Since the ultimate aim of these experiments was to study the reaction under pressure, the following procedure was designed to keep the enzyme and substrate separate until used under pressure and at various temperatures. With semiautomatic, syringe pipettes (to speed the procedure and avoid changes in the trypsin) a set of test-tubes is charged with 3.0 ml. each of 5 per cent TCA. To each of a set of stainless steel bombs (Johnson, Baylor, and Fraser (2)) are added the above casein solution, a stainless steel messenger, and 5 drops of hexadecane (Paragon). (The hexadecane (m.p. 18°) may be replaced by tetradecane for experiments at lower temperature.) Both have the advantages of inertness, availability, and lack of effect on the optical density when removed as described. Hexadecane melts and allows mixing within 1 minute, even at 25°, and in glass tubes.) The bombs are chilled in ice until the hexadecane is frozen (3 to 5 minutes) and meanwhile the trypsin solution is prepared. Then 1.1 ml. of the fresh trypsin solution are added and enough hexadecane to fill the bomb (2 to 5 drops). A rubber dam is placed on each bomb, the top is screwed in place, and the group is immersed in the bath at the desired temperature. After 1 minute the set of bombs is removed, inverted several times to allow the messengers to mix substrate and enzyme, giving a final pH of 7.1, and then returned to the bath. At the desired time intervals the bombs are removed, and the contents dumped into the TCA solution and poured back and forth. After at least 1 hour the mixture is again chilled to solidify the hexadecane and filtered, and the optical density is determined at room temperature at 280 m μ in the Beckman quartz spectrophotometer. The optical density is converted to per cent of casein undigested by using the calibration curve of Fig. 5, the preparation of which is described below.

Calibration—Kunitz, using the optical density of a TCA filtrate as above, devised a method for the determination of trypsin activity by measuring the casein digested in a given time (8). Here, however, we must know the amount or fraction of casein digested. To relate casein digested to

optical density, varying amounts were digested completely (under the stated conditions this requires about 3 hours, but the digestions were actually run for 18 to 18.5 hours to insure completion) and the optical density of the filtrate was measured. To compensate for the varying charges of casein half the usual volume of 10 per cent (instead of 5 per cent) TCA solution was used for quenching and an equal volume of buffer (which has no effect on the optical density) containing enough casein to make up the charge differential was added before the experimental sample.

Completion of casein digestion implies partial subsequent digestion of the trypsin by itself. If the same amount of casein is digested completely with varying amounts of trypsin, the variations in optical density must be

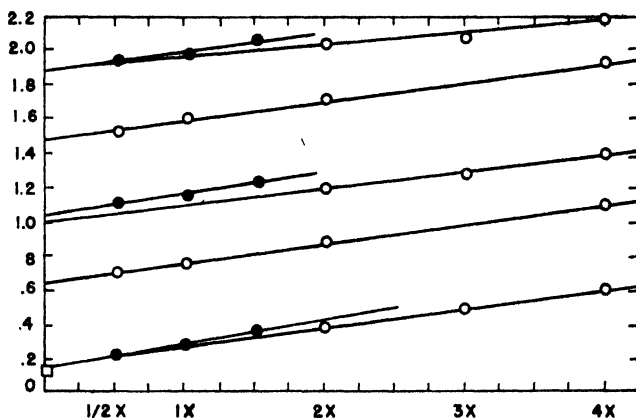


FIG. 5. Complete digestion of varying amounts of casein with varying amounts of trypsin. Ordinate, optical density at 280 $m\mu$; abscissa, trypsin concentration. $X = 0.212$ mg. per ml. of final concentration. 100 per cent casein = 5.77 mg. per ml. O, ●, different experiments.

due to the self-digested trypsin (since undigested trypsin has no effect on the blank). Hence, by extrapolation back to zero concentration of trypsin, one can estimate the optical density which would have been produced from casein alone. This was done with the results shown in Fig. 5, and the resulting intercepts were used to make the calibration of Fig. 6.

Since the zero intercept for casein did not quite coincide with the blanks prepared by adding trypsin and casein separately to TCA, the straight line of Fig. 6 was drawn for the best fit to these points and the blank average. The error possibly introduced in this compromise is insignificant in all except the ^{14}C -T experiments.

Discussion of Methods—In considering the results of experiments in which the kinetics are based on determination of some property which varies with the time of reaction, it must always be questioned whether the

quantity measured is a true indication of the disappearance of the reactant. In other words, we are determining here the rate of *appearance* of a somewhat arbitrarily defined product and considering it as a measure of the *disappearance* of casein. In justification three observations may be pointed out: First, it has been found that the optical density curve (Fig. 6) follows Beer's law; *i.e.*, it is linear. Secondly, the opalescence produced by TCA with most digested casein disappears at the same time that the optical density of the filtrate reaches a maximum. Since the precipitate is an indication of undigested casein or large fragments, this means that no precipitable, undigestible fragments are piling up; on the other hand, once the precipitate disappears, the optical density, which is our measure of casein disappearance, no longer increases. Thirdly, in the preparation of the

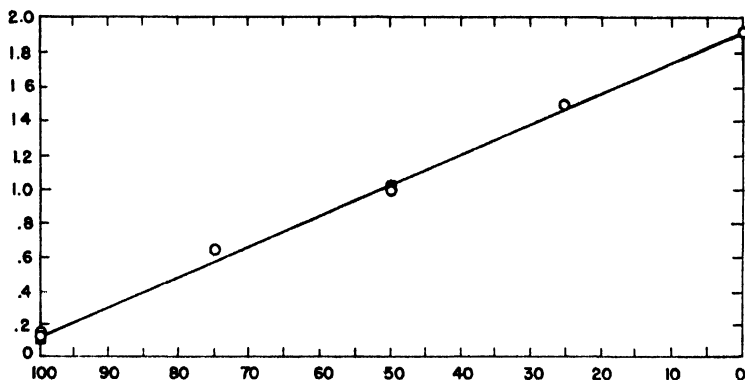


FIG. 6. Calibration of casein digestion. Ordinate, optical density at 280 mμ; abscissa, per cent of casein undigested. 100 per cent casein = 5.77 mg. per ml. The points are the zero extrapolations of Fig. 5. □, blank experiment.

calibration of trypsin self-digestion (Figs. 1 and 2), the drop in digestive activity was followed simultaneously with the appearance of the optical density of the filtrate. The two processes were completely parallel within the limits of experimental error; *i.e.*, if the per cent of active trypsin determined by ability to digest casein is plotted against the optical density of the self-digested filtrate, a straight line is obtained which passes through the blank. The increase in optical density is thus a measure of the disappearance of the protein trypsin as manifested by its enzymatic activity.

In previous studies of casein-trypsin digestion various properties have been used as the measure of the amount digested. Some (*e.g.* Van Slyke amino nitrogen or formol titration) actually measure the number of bonds cut; others (*e.g.* viscosity) measure protein disappearance, but are difficult to interpret. The criterion of a satisfactory property is that it measures the appearance or disappearance of a substance on one or the other side

of the rate-determining step and that other kinetic processes do not excessively complicate its increase or decrease. The present assumption is that TCA precipitation discriminates between molecules of the size of casein and considerably smaller particles which are either the direct result of the rate-determining step or which are formed very rapidly thereafter. This presumption is strengthened not only by the above observations but also by the reasonable nature of the results.

DISCUSSION AND RESULTS

Although the digestion of casein by trypsin has been extensively studied, no satisfactory picture of the process has appeared. In view of the conflicting results in the literature, it seems necessary to start by showing that the casein-trypsin digestion cannot be explained by a simple classical rate law, and then to proceed to more elaborate considerations.

The simplest picture of casein-trypsin digestion is one in which trypsin self-digestion is negligible; *i.e.*, the trypsin is a true catalyst. A set of appropriate plots for the C-T data shows that by the usual techniques of kinetic analysis the data fit either the first or second laws with a little straining. In each case, however, the best straight line fails to pass through the zero intercept by a considerable margin. When the initial trypsin concentration is varied, a rather clear linear dependence on concentration can be seen. But when the starting concentration of casein is varied, the conclusion is reached that the reaction is apparently zero order for casein. Since this is completely inconsistent with the results of individual runs and with the results of enzymatic kinetics in general, it is obvious that this interpretation is faulty.

The lack of agreement of the simple kinetic models with the data can be more clearly shown by use of the slide-fit technique, and it may be seen from the casein digestion points of Fig. 3 that the rate falls off even faster than the most improbable third order.

Rôle of Trypsin Self-Digestion—The most striking feature of all of the data (Figs. 7 and 8) is the extremely rapid dropping of the rate as the reaction proceeds. This aspect of casein-trypsin digestion has been noted before (9) by other investigators and various reasons have been assigned to it.

Since it became known that trypsin exists in dynamic equilibrium with a reversibly denatured trypsin (4) and that the active form is capable of digesting the denatured form, it has frequently been assumed that the complexities of trypsin digestions are due in part, at least, to concomitant self-digestion of the enzyme. Northrop has shown (10) that casein digestion at 0° is a first order process and it was believed that this was because of the lack of self-digestion at this temperature. The data for trypsin self-

digestion (Section I) show that the rate is slower than the casein-trypsin reaction by about an order of magnitude in the concentration range which we are considering. It is *a priori* unlikely, therefore, that enzymatic self-digestion is important in the rate decline of casein-trypsin digestion. Ac-

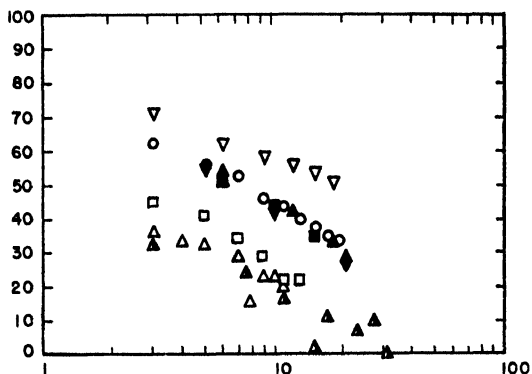


FIG. 7. α C-T raw data. Varying amounts of casein digested with the same amount of trypsin. Ordinate, per cent undigested; abscissa, time in minutes (logarithmic scale). $\frac{1}{4}$ C-T (Δ , Δ); $\frac{1}{2}$ C-T ($\square$); C-T ($\circ$); 2C-T (∇). The solid symbols are the corresponding controls.

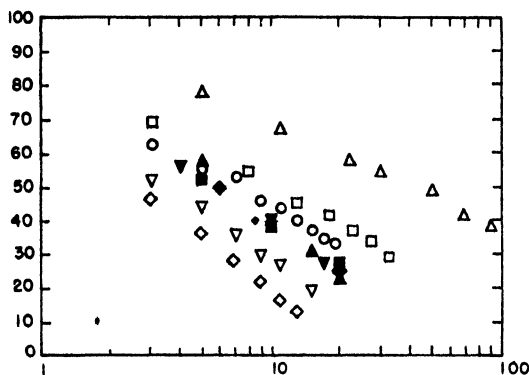
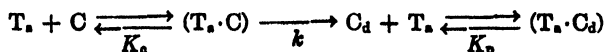


FIG. 8. C- α T raw data. The same amount of initial casein digested with varying amounts of trypsin. Ordinate and abscissa as in Fig. 5. C-1/10T (Δ); C- $\frac{1}{2}$ T ($\square$); C-T ($\circ$) (same points as in Fig. 5); C-2T (∇); C-3T ($\diamond$). The solid symbols are the corresponding controls.

tually the rate change introduced even by assumption of trypsin self-digestion (at its normal rate in the absence of casein) turns out to be inconsiderable, even at extreme degrees of reaction (see the dotted curve in Fig. 9). The possibility of the rate drop being due to self-digestion of the enzyme can, therefore, be dismissed.

Inhibition by Reaction Products—In the literature on casein-trypsin, mention has frequently been made of the inhibiting effect of the products of the reaction. Although later kinetic studies seem to have neglected this factor, Northrop (7) has investigated this effect, using partially purified trypsin, and has shown that the inhibitor combines reversibly with the trypsin and that the ratio is probably 1:1.

Assumption of the mechanism



allows substitution in the rate equation

$$-\frac{dC}{dt} = k(T_s \cdot C) = kK_s(T_s)(C)$$

in a manner completely parallel to that used in the trypsin system. We need not consider the equilibrium between T_s and reversibly denatured trypsin because this is rapidly attained (4) and has no effect on the kinetics of the digestion process. Trypsin self-digestion is also disregarded because it has been shown above to be too slow to be of significance. In the substitution for T_s , the following equation is obtained (cf. Section I).

$$(T_s) = \frac{(T_s \cdot C_d) + (T_s) + (T_s \cdot C)}{1 + K_p(C_d) + K_s(C)}$$

Actually by comparing the complete integrated form with the data, even assuming extreme cases, we have found that the amount of T_s not combined with the product is arithmetically negligible compared to that in the form $T_s \cdot C_d$. To simplify the development this factor is introduced and the variable α , the degree of reaction, is substituted, as was done in the trypsin derivation. Then

$$(C_0) \frac{d\alpha}{dt} = kK_s \frac{(T_0)(1 - \alpha)(C_0)}{K_p \alpha(C_0)}$$

which on integration gives

$$\ln \left(\frac{1}{1 - \alpha} \right) - \alpha = \frac{kK_s}{K_p} \cdot \frac{(T_0)}{(C_0)} \quad (6)$$

This cannot be solved for α and tested in the usual manner, but comparison of this rather complicated relationship with the data may easily be made by means of slide-fit technique.

Comparison of Data and Theory—The raw data are plotted in two sets (Figs. 7 and 8) to emphasize the dependence on the starting concentrations of casein and of trypsin and to avoid confusion of the points. It can be

seen from Equation (6) that the data for all experiments should, if the hypothesis is correct, be representable by a single curve, provided that the time values for various experiments are displaced by the appropriate values

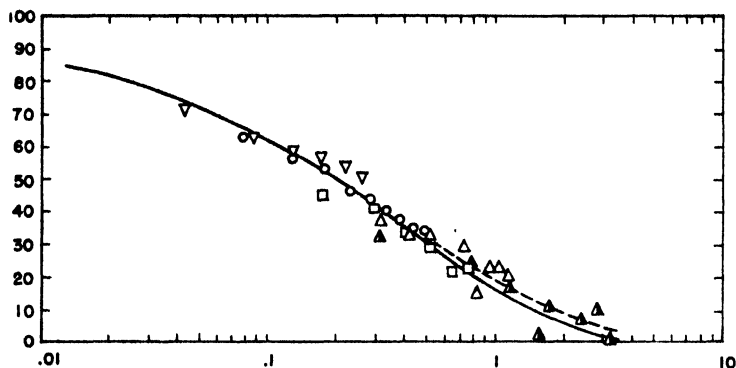


FIG. 9. α C-T data of Fig. 7 displaced for control trypsin data and initial casein charge and fitted by slide-fit technique to theoretical curves describing first order inhibition of the trypsin by the products of digestion. Ordinate, per cent undigested; abscissa, theoretical rate integral, logarithmic scale (see the text). The dotted curve shows the alteration introduced by assumption of trypsin self-digestion at a normal rate.

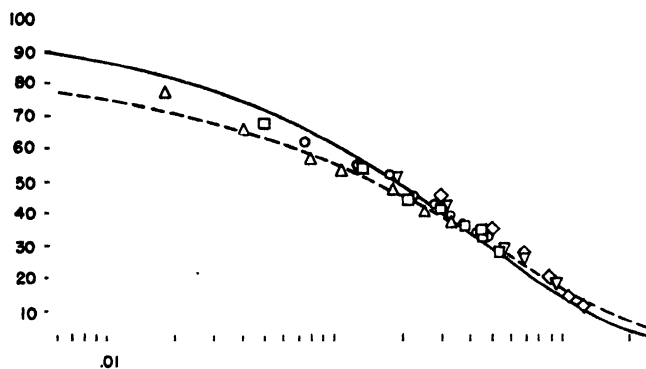


FIG. 10. C- α T data of Fig. 8 displaced for initial trypsin charge and fitted by slide-fit technique to same theoretical curve used in Fig. 9. Ordinate and abscissa as in Fig. 9. The dotted curve shows the fit of a theory which assumes the casein to contain 15 per cent of a more rapidly digested component.

of T_0 and C_0 . T_0 cannot, because of the instability of trypsin solutions, be simply calculated from the dilution of the stock. A control digestion with standard T_0 and C_0 is therefore run with each experiment and the correct T_0 value determined by the best fit of the control points (see Figs. 7 and 8) to the curve of the standard run, which is taken arbitrarily as having unit charges.

The standard (C-T) data are then transferred to another sheet of graph paper; the new sheet is moved horizontally by the corrected $T_0:C_0$ ratio for each experiment and the points for this experiment traced. The results are again shown in Figs. 9 and 10 for clarity.

Now the assembled data can be fitted to the theoretical curve obtained in the above derivation by superposition of the sheets and sliding along the x axis. It can be seen (Figs. 9 and 10) that the fit of the data is fairly good, especially considering the wide range of the reactant ratio, and much better than that obtained by any previous hypothesis. The discrepancies occur in the early points which lie below the theoretical curve, and, in general, it seems that the fit might be better if the curve were somewhat flatter.

Second Order Inhibition—The most obvious effect which would result in a flatter curve is the assumption of a higher order of inhibition. Actually the curve for second order inhibition is not greatly different from the above theoretical curve and results in a better fit to some of the data. Such a hypothesis does not seem readily interpretable mechanistically. Moreover, this is so clearly inconsistent with the trypsin self-digestion data and with Northrop's experience (7) with the inhibitor that it was abandoned.

Other Inhibition Effects—In the above derivation several factors were omitted. If we consider that active trypsin may be present in appreciable amount or that an arithmetically significant amount may be bound by itself and by undigested casein, corresponding integrated equations may be obtained, including as many such factors as desired. All such equations, however, lead to theoretical curves steeper than those of Figs. 9 and 10, and, in all cases, a less satisfactory fit by far, thus justifying their omission in the above derivation.

In passing it may be noted that several other hypotheses were tested to be sure that the data could not be equally well fitted. They include assumption of a varying order of inhibition, depending on the ratio of casein to trypsin, assumption of second order inhibition with trypsin or unchanged casein, assumption of higher orders of inhibition with the product, and assumption of a sum of first and second order inhibition with the product. The failure of any of these, even in extreme cases, to give at all satisfactory fits lends confidence to the view that the above explanation is valid.

Comparison with Previous Work—Not all the data which were obtained by Northrop on the digestion of casein are comparable, since much of the early work was done with partially purified enzyme and led to anomalous results. With crystalline trypsin Northrop (9) digested casein under conditions similar to ours, determining the digested casein by TCA precipitation and nitrogen analysis of the supernatant fluid. In Fig. 11 Northrop's data from the three experiments at different concentrations of casein

digested at 35° have been fitted to the same theoretical curve which was used in Figs. 9 and 10. The fit lends confidence to both the present theory and analytical method.

Inhomogeneity of Casein—One of the points which it was of interest to consider in connection with the anomalies noted in casein-trypsin digestion by previous workers was the known inhomogeneity of casein. Examination of casein electrophoretically has indicated that so called purified casein, prepared by isoelectric precipitation, as was the casein used in the present work, consists of two main fractions in the ratio 81:19 and is subject to small variations depending on the source (11).

The data of Figs. 9 and 10 lend themselves readily to the assumption that the casein is not pure, in the sense that a minor component has been

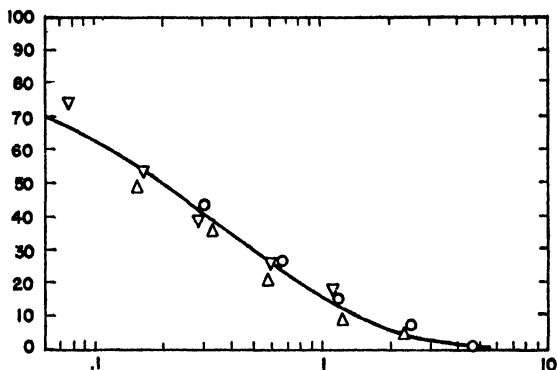


FIG. 11. Data of Northrop fitted to the curve of Figs. 9 and 10. Same ordinate and abscissa.

digested much more rapidly than the main fraction. It may be seen that the data tend to extrapolate to about 85 per cent instead of 100 per cent. (This value was actually determined by means of a log *versus* log plot, which shows the effect much more clearly.) If this is true, the early part of the digestion should show characteristic behavior and drop rapidly from 100 per cent to the lower curve. In Fig. 10 (dotted curve) and Fig. 12 the assembled data have been fitted to a theoretical curve, each point of which is 85 per cent of the corresponding ordinate of the curve of Figs. 9 and 10. In addition, in Fig. 12 further data³ are included, covering the first part of the reaction and showing that the fit to the original curve is reasonably satisfactory. With the curve of 85 per cent pure casein, however, the behavior is exactly that which would be expected. It is of interest to note that these data were obtained in a different laboratory by

³ These additional data were obtained by Mr. A. C. Knight, Department of Chemistry, University of California.

another investigator using different casein and a new sample of trypsin (from the same original manufacturer's lot) and using an altered technique in which the samples were taken from a single batch stirred at 35°. Considering that these samples could include no controls to link them to the previous data, the fit and overlap are excellent.

A theory to cover the whole range could be constructed and handled by the slide-fit technique, but would be of little value since it would involve the introduction of additional assumptions and several new parameters which could be evaluated only roughly, because of the relatively minor importance of the rapidly digested component and the relative closeness of its rate to that of the major component.

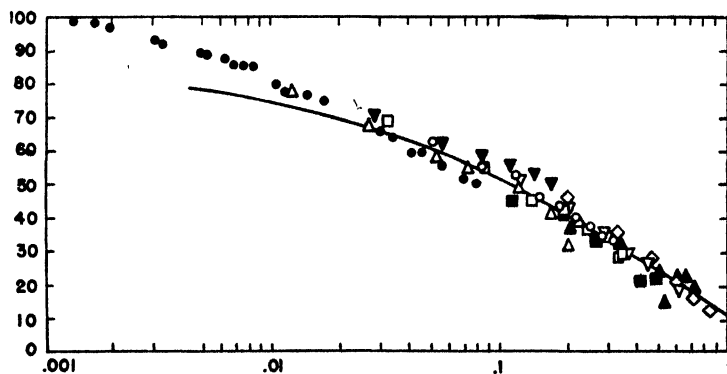


FIG. 12. Data of Figs. 9 and 10 plus further data covering the early stages of digestion fitted to a curve which assumes casein to contain 15 per cent of a more rapidly digested component. The solid symbols are the α C-T data, the open symbols are the C- α T data, the solid circles the added data. Same ordinate and abscissa.

Qualitatively, however, it may be said that casein behaves as a mixture and that the proportions of the components determined kinetically are strikingly close to those obtained independently from electrophoretic measurements, probably within the variations in the sources. The minor component digests approximately 5 times as fast as the major component. The approximate rate constant for the major fraction is 0.018. It must be remembered that these are portmanteau constants and include several equilibrium constants, as well as the specific rate constant of the digestion.

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data included in Fig. 12. Particular appreciation is due Miss Rosamond S. Baker for conscientious and capable technical assistance.

SUMMARY

Preliminary data for the self-digestion of trypsin and more complete data for the digestion of purified casein by crystalline trypsin are presented and analyzed. Both reactions have been studied in phosphate buffer, pH 7.1, at 35°, and the casein data cover a range of 97 per cent completion and a concentration ratio of 160 (casein to trypsin). The data are representable by a single parameter expression in each case on the assumption that the reaction involves 1 molecule of trypsin and 1 of casein (or trypsin), but that the enzymatic activity of trypsin is inhibited by a reversible equilibrium with the products of digestion. A closer fit to the casein data is obtained by the further assumption that the casein consists of two components digested at different rates; the proportions obtained from the present kinetic data agree well with the known composition of casein. Comparison of the data to the theory is made by a graphical method of analysis which is generally applicable to kinetic data and most useful in cases in which the integrated rate law is complex.

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THE OCCURRENCE OF A WIDE VARIETY OF TRANSAMINASES IN BACTERIA*

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The occurrence and general importance of transaminases for the formation of amino acids have been variously discussed since the discovery of these enzymes by Braunstein and Kritzmann (1) more than 10 years ago. The initial report indicated that most, if not all, of the monocarboxylic amino acids undergo transamination with α -ketoglutarate or oxalacetate as the amino acceptor. This view was challenged by Cohen (2) who, working with animal tissues, observed transaminase activity only for the pairs, glutamic-aspartic and glutamic-alanine. Although the methods available at the time of Cohen's work lacked specificity and were less sensitive than desired, examination of his data indicates in retrospect that several other amino acids participate in transamination. To date two transaminases have been demonstrated in animal (3) and bacterial (4, 5) cells and have been studied in detail after separation and purification. Both of these, the glutamic-aspartic and glutamic-alanine systems, have been rather highly purified from animal tissues (3) and, subsequently, pyridoxal phosphate has been shown to act as coenzyme (5, 6).

The occurrence of a larger variety of transaminases has been suggested by three types of studies: (a) the substitution of the α -keto or α -hydroxy analogues for various essential amino acids for both animals (7-10) and microorganisms (11-13); (b) isotopic studies in which N^{15} , fed as ammonium ion, or tyrosine is found distributed in the α -amino group of several amino acids (14, 15); and (c) direct studies of the transfer of amino groups to ketoglutarate in yeast (16). These investigations and a discussion of the present status of the transamination reaction are reviewed in more detail by Gunsalus (17).

The advent of paper chromatographic methods and the availability of specific amino acid decarboxylases afforded an opportunity to clarify the knowledge of the occurrence of transaminases and of their possible relationship to amino acid formation.

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In the present study, in order to determine whether the number of amino acids participating in transamination reactions is greater than those recognized, several types of bacteria have been grown under a variety of conditions and examined for transaminases. As indicated in a preliminary report (18), dried cell preparations of *Escherichia coli*, *Pseudomonas fluorescens*, and *Bacillus subtilis* catalyze the formation of glutamate in the presence of α -ketoglutarate as amino acceptor and the following amino acids as amino donors: aspartate, alanine, valine, leucine, norleucine, tryptophan, tyrosine, phenylalanine, and methionine. Slight transaminase activity can also be observed with isoleucine, histidine, lysine, threonine, and glycine. The amino acids were identified by paper chromatography, and more quantitative data for two of these transaminases were obtained manometrically with specific amino acid decarboxylases.

The glutamic-tyrosine and glutamic-phenylalanine transaminases have been obtained in extracts of *E. coli* (B), and both the dried cell preparations and the cell-free extracts have been shown to require pyridoxal phosphate for maximum activity.

Methods

Culture—*E. coli*, strain B, from Dr. S. E. Luria's culture collection, was grown in his Medium S2, which contains, per liter, KH_2PO_4 , 1.5 gm.; Na_2HPO_4 , 13.5 gm.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 gm.; CaCl_2 , 0.01 gm.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0005 gm.; NH_4Cl , 2.0 gm.; and glucose, 4 gm. The culture was incubated with shaking for 13½ hours at 30°, and harvested by centrifugation in a Sharples centrifuge. The cells were washed by suspending them in 200 ml. of distilled water and again centrifuging. The washed cells were suspended in 20 ml. of water and dried *in vacuo* over Drierite. The lyophilized cells, approximately 7 gm. per 10 liters of medium, were stored in vials at -20°.

A culture of *P. fluorescens*, strain A3.12, grown in an ammonium medium containing mandelate as an energy source, was kindly furnished by Dr. R. Y. Stanier (19). *B. subtilis*, Marburg strain, was harvested from the synthetic medium of Hyndman (20).

Cell Extracts—To extract the enzymes, 5 gm. of vacuum-dried cells were suspended in 50 ml. of 0.1 M phosphate buffer, pH 8.3, along with 2.5 gm. of No. 500 carborundum, and treated for 1 hour in a 50 watt 9-KC Raytheon magnetostriction oscillator. The cellular debris was removed by centrifugation and the activity of the clear supernatant fluid studied. The enzymes in these extracts were stable when stored at -20° for a period of several months.

Reagents— α -Ketoglutarate, prepared by the method of Friedman and

Kosower (21), or later purchased from the Bios Laboratories, Inc., was used as amino acceptor in the majority of these experiments. L- or DL-amino acids purchased from Winthrop or General Biochemicals were used throughout. The purity of these amino acids was checked by their behavior on paper chromatograms, under conditions in which contaminations large enough to yield a level of glutamate measurable in the transamination experiments would have been detected. Whenever DL mixtures were used, it was assumed that only the L-amino acid was active in transamination; thus the substrate concentration was doubled.

Pyridoxal phosphate, prepared according to the method of Gunsalus *et al.* (22), is expressed in terms of micrograms of material with a purity index of 1 (approximately 90 per cent purity).

Assay for Transaminases—The formation of glutamate was demonstrated by means of ascending paper chromatography. For the systems described, a mixture containing 0.1 ml. of 1 M phosphate buffer, pH 8.3, 0.1 ml. of 0.5 M α -ketoglutarate, 0.2 ml. of 0.125 M L-amino acid, 0.2 ml. of cells, 50 mg. per ml., pyridoxal phosphate or other additions, and water to 1 ml. was incubated for 60 minutes at 37°. The reaction was stopped by the addition of 0.1 ml. of 100 per cent trichloroacetic acid, and 0.02 ml. was transferred to Whatman No. 3 filter paper sheets approximately 15 × 18 inches. After the spots had dried, the paper was suspended in a chamber containing ammonia vapors to neutralize the acid salts and liberate the free amino acids. The paper was then transferred to a chamber containing 200 ml. of water-saturated phenol, and the solvent allowed to ascend at 25° until it had moved to within 2 inches of the top of the paper. The chromatograms were dried in an oven at 90° for 10 minutes, sprayed with ninhydrin (0.25 mg. per ml. of water-saturated butanol), and again heated at 90° for 5 minutes. Chromatograms were read visually in comparison with known quantities of amino acids, the individual amino acids being identified by their R_f values as shown in Figs. 1 to 4.

For the manometric determination of transamination, 1 ml. of the reaction mixture described above was incubated for the desired time at 37°. The reaction was stopped by placing the tube in boiling water for 5 minutes (separate experiments demonstrated that non-enzymatic transamination did not occur during heating), the pH adjusted to 5.0, and 1 ml. transferred to the main cup of a Warburg vessel containing 1 ml. of 0.075 M phthalate buffer, pH 5.0. 0.5 ml. of a 10 mg. per ml. suspension of dried cells containing the specific decarboxylase was added to the side arm. After 10 minutes equilibration at 37°, the contents of the side arm were tipped in and the evolution of CO₂ followed until the decarboxylation was complete.

EXPERIMENTAL

The results of transamination with dried cells of *E. coli* (B) as depicted in filter paper chromatograms are shown in Figs. 1 to 3. Various amino donors were incubated with α -ketoglutarate, and the appearance of glutamic acid was interpreted as an indication of a transamination reaction.

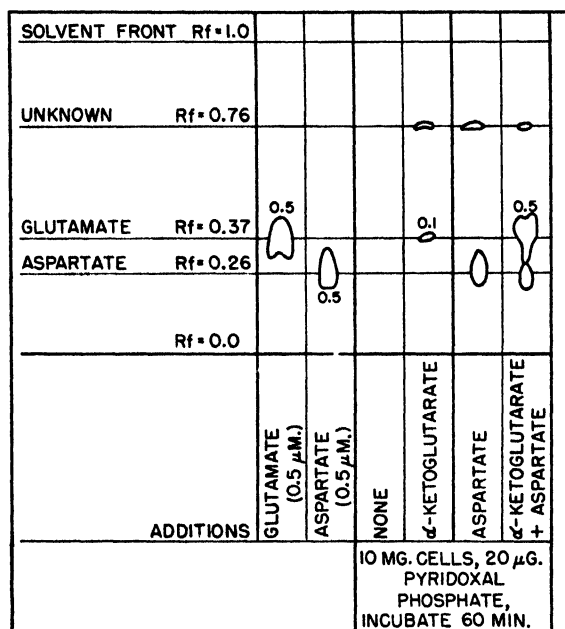


FIG. 1. Glutamic-aspartic transaminase in *E. coli* (B). The reaction mixture, incubated for 1 hour at 37°, contained 10 mg. of dried cells and 20 γ of pyridoxal phosphate, with other additions as indicated. The numbers above and below the spots in Figs. 1 to 4 are the micromoles of the amino acid on the chromatogram, as estimated from the size and density of the spot in direct comparison to known amounts.

The control experiments and those demonstrating the glutamic-aspartic transaminase are shown in Fig. 1. As indicated, experiments lacking the amino donor showed only a trace of glutamate, while those lacking other reactants singly, including α -ketoglutarate, showed no glutamate formation. The complete system in which the reaction was stopped at zero time also showed no glutamate. However, when aspartic acid and α -ketoglutarate were incubated with the enzyme (dried cells) for 1 hour, a large quantity of glutamic acid was formed, indicating the presence of an active glutamic-aspartic transaminase.

The results of the chromatographic investigation for other transaminases

bacteria. At the present stage of knowledge, glutamate is shown to be formed during incubation of α -ketoglutarate with each of the amino acids mentioned as amino donor. For more complete knowledge, each of these systems must be studied in detail to determine the exact reaction involved. The absence of glutamate formation in the presence of ammonia, or ammonia plus glucose as hydrogen donor, further substantiates the fact that transamination is involved.

Additional data on the nature of the aromatic amino acid transaminases have been obtained by a chromatographic study of the glutamic-tyrosine and glutamic-phenylalanine reactions with a cell-free extract from *E. coli*

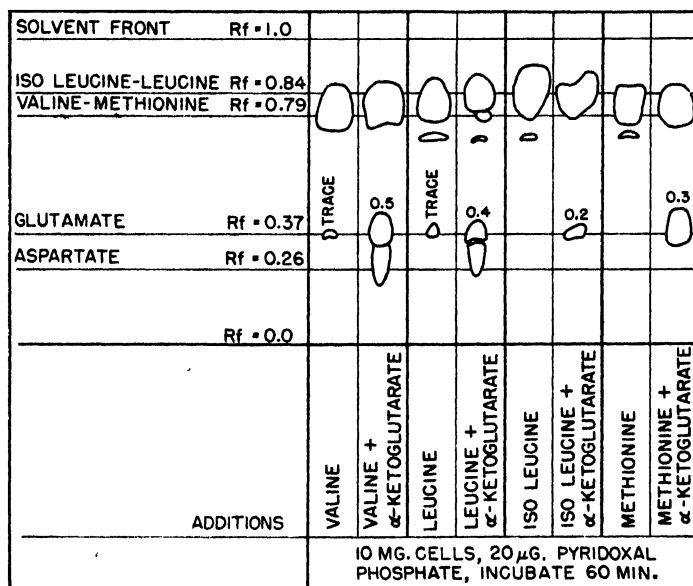


FIG. 3. Transaminase present in *E. coli* (B). Conditions as for Fig. 1

(B). The reversibility of these reactions has been shown by incubating α -ketoglutarate with tyrosine and phenylalanine as amino donors, as well as by incubating α -ketotyrosine (*p*-hydroxyphenylpyruvic acid) and α -ketophenylalanine (phenylpyruvic acid) with glutamate as amino donor (Fig. 4).

Quantitative data on the aromatic amino acid transaminases were obtained with cell-free extracts by use of the glutamic and tyrosine decarboxylases for analysis. The conditions for these experiments are shown in Table II. The cell-free enzyme used had been precipitated twice with ammonium sulfate and in the process resolved with respect to pyridoxal phosphate. As suggested in Table II, the glutamic-tyrosine and glutamic-

phenylalanine transamination reactions proceed at a good rate in both the forward and reverse directions. In studying the glutamic-tyrosine transaminase, glutamate formation was determined with glutamic decarboxylase, and tyrosine formation with tyrosine decarboxylase. For the glutamic-phenylalanine transaminase, glutamate formation was measured in the same manner; however, in the reverse reaction, glutamate

TABLE I
Transaminases Present in Bacteria

Conditions, 50 μ M of α -ketoglutarate, 25 μ M of amino acid, 10 mg. of dried cells; 1 ml. volume. Analysis, filter paper chromatogram, 0.02 ml. sample; solvent, water-saturated phenol.

Amino acid donor	$R_f \times 100$	Glutamic acid formed in 60 min.		
		<i>E. coli</i> (B)	<i>P. fluorescens</i> (A3.12)	<i>B. subtilis</i> (C4)
		μ M	μ M	μ M
Aspartic acid	23 $\pm$ 2	25	25	20
Alanine	61 $\pm$ 2	0	12	5
Valine	77 $\pm$ 2	25	15	10
Leucine	83 $\pm$ 1	20	25	10
Isoleucine	84	10		
Norleucine	81 $\pm$ 1	20	10	
Tyrosine	66	12	12	Trace
Phenylalanine	84 $\pm$ 2	15	13	0
Tryptophan	75 $\pm$ 3	15	15	0
Methionine	80	15	20	Trace
Threonine	48 $\pm$ 1	8	10	"
Lysine	74	10	10	0
Histidine	75 $\pm$ 1	10	10	10
Glycine	44	10	10	Trace
Proline	86 $\pm$ 2	0	0	0
Hydroxyproline	67	0	0	Trace

disappearance in the presence of α -ketophenylalanine was used as the criterion of transamination. A more thorough investigation of the aromatic amino acid transaminases is being carried out and will be reported later.

These observations show that the level of the glutamic-tyrosine and glutamic-phenylalanine transaminases is sufficient to account for the formation of these amino acids during growth, thus further indicating that transamination is of broader importance in the synthesis of amino acids

than has been previously recognized. The problem indicated by the work of Simmonds, Tatum, and Fruton (24), in which the ketotyrosine replacement of tyrosine for a *tyrosineless* mutant of *E. coli* required approximately 100 times as much ketotyrosine, suggests that (a) transamination is not on the route of tyrosine formation; (b) the substrate is not readily available to the enzyme; and (c) ketotyrosine is less stable in the presence of the bacterial enzymes than is tyrosine.

SOLVENT FRONT	Rf = 1.0								
PHENYL ALANINE	Rf = 0.84				0.4			1.0	0.5
TYROSINE	Rf = 0.67	D		0		1.0	0.5	D	
				0.4					
GLUTAMATE	Rf = 0.37	0.1	1.0	0.5	0.5		0.5		0.5
	Rf = 0.0								
ADDITIONS	α -KETOGLOUTARATE								
	GLUTAMATE								
	GLUTAMATE + α -KETOTYROSINE								
	GLUTAMATE + α -KETOPHENYLALANINE								
	TYROSINE								
	TYROSINE + α -KETOGLOUTARATE								
	PHENYLALANINE								
	PHENYLALANINE + α -KETOGLOUTARATE								
CELL-FREE EXTRACT + 20 μ G. PYRIDOXAL PHOSPHATE, INCUBATE 60 MIN.									

FIG. 4. Tyrosine and phenylalanine transaminases in cell-free extract of *E. coli* (B). The reaction mixture, incubated for 1 hour at 37°, contained the cell-free enzyme, 20 γ of pyridoxal phosphate, 50 μ M of α -keto acid (when added), and 25 μ M of L-amino acid (when added), as indicated on the figure.

In these studies, it has been found that the glutamic-tyrosine transaminase activity of the dried cells is 3 times that shown by the resting cells from which they are prepared. Thus it appears that the larger ketotyrosine requirement arises in part from permeability factors. The lesser stability of the ketotyrosine may also contribute to the decreased activity in the growth experiments.

A recent report of Tanenbaum and Shemin (25) suggests the presence of a glutamic-leucine transaminase in pig heart, and work by Housewright and Thorne (26) indicates the presence of a glutamic-phenylalanine transaminase in *B. subtilis*.

Until these reactions and those reported in this paper are studied in purified systems, it is not possible to determine the precise route of reaction. In view of the report of Braunstein (27) of an aspartic-alanine transaminase, which appears to be the result of the summation of the glutamic-aspartic and glutamic-alanine systems, as indicated by O'Kane and Gunsalus (28), one must obtain more complete data before reaching definitive conclusions on the reaction routes.

TABLE II

Glutamic-Tyrosine and Glutamic-Phenylalanine Transaminases in E. coli (B)

Conditions, 50 μ M of α -keto acid, 25 μ M of L-amino acid, 20 γ of pyridoxal phosphate as indicated, cell-free enzyme; 1 ml. volume; pH 8.3. Analysis, glutamate, glutamic decarboxylase, tyrosine, tyrosine decarboxylase.

ditions			Amino acid formed in 60 min.		Resolution per cent
			Glutamate μ l.	Tyrosine μ l.	
Tyrosine					
α -Ketoglutarate	+	pyridoxal phosphate	9		
"	+	tyrosine	25		
"	+	"			
"	+	pyridoxal phosphate	124		80
α -Ketotyrosine	+	"		20	
"	+	glutamate		246	
"	+	pyridoxal phosphate			
Phenylalanine					
α -Ketoglutarate	+	phenylalanine	9		
"	+	"	191		94
		pyridoxal phosphate	Used		
		phenylalanine	0		
		"	55		
		pyridoxal phosphate	182		

SUMMARY

1. By use of dried cells of *Escherichia coli*, *Pseudomonas fluorescens*, and *Bacillus subtilis*, a series of transaminases has been demonstrated which produces glutamic acid from α -ketoglutarate with the following amino donors: aspartate, alanine, valine, leucine, norleucine, tryptophan, tyrosine, phenylalanine, and methionine, and, to a lesser degree, with isoleucine, histidine, lysine, glycine, and threonine.

2. The glutamic-tyrosine and glutamic-phenylalanine transaminases have been obtained in cell-free form and pyridoxal phosphate has been shown to act as coenzyme.

3. Manometric data demonstrating the reversible nature of the aromatic amino acid transaminases are presented.

4. Further studies of purified systems are indicated in order to determine the route of reaction and the possible importance of these reactions in the biosynthesis of amino acids.

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MANOMETRIC DETERMINATION OF METHANOL IN THE PRESENCE OF ETHANOL IN BLOOD

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Numerous methods, based on a variety of reactions (1-6),¹ are suitable for the determination of methyl alcohol in the presence of ethyl alcohol in aqueous solutions, including distillates of deproteinized filtrates of blood, tissues, etc. The procedure to be described is an extension of that previously reported in abstract (7) and is based on the bichromate-sulfuric acid oxidation of alcohols in aqueous solution, which may be conducted so that ethyl alcohol yields acetic acid, whereas methyl alcohol is quantitatively converted to carbon dioxide (1). By performing the oxidation in a closed system, similar to one of those employed in the determination of α -amino carboxyl CO_2 (8), the carbon dioxide liberated may be readily transferred to the chamber of the Van Slyke-Neill manometric gas analyzer and absorbed by alkali, with subsequent acid decomposition of the carbonate and measurement of the pressure of the CO_2 liberated before and after absorption (9). Blood is prepared for analysis by distilling a tungstate filtrate, essentially as described by Harger for the determination of ethyl alcohol (10).² The amount of blood required is usually 3 ml., but smaller quantities suffice when high concentrations of methyl alcohol are present.

The method is particularly convenient when only occasional analyses are to be performed, as it is independent of standard solutions, and requires only one simple accessory in addition to the usual equipment maintained with the Van Slyke-Neill analyzer and the Harger distillation apparatus. The specificity features of bichromate oxidation applied to distillates of blood, urine, and tissues for the determination of alcohols have been subjects of many discussions (11, 12). It may be concluded that in the absence of significant concentrations of formaldehyde and formate or acetoacetate, associated with severe ketonemia, the carbon dioxide liberated may be assumed to have arisen from methyl alcohol. In routine forensic testing, the qualitative chromotropic acid test for formaldehyde derived

¹ Personal communication from Dr. R. N. Harger with excerpts from a paper listed as to be published in the near future.

² Tungstate filtrates of tissues homogenized in a Waring blender are also suitable for analysis.

from methyl alcohol (*e.g.*, as described by Ozburn (13)), employed prior to the quantitative determination, serves as a partial check upon the possibility that the carbon dioxide comes from some compound other than methyl alcohol. In our experience recoveries of methyl alcohol added to blood containing various concentrations of ethyl alcohol (see below) range from 98 to 101 per cent.

Method

Apparatus—

1. The Van Slyke-Neill manometric gas analyzer and accessories, as described by Van Slyke *et al.* (8) for the determination of carboxyl CO_2 . "Apparatus C" there described is modified in chamber dimensions and

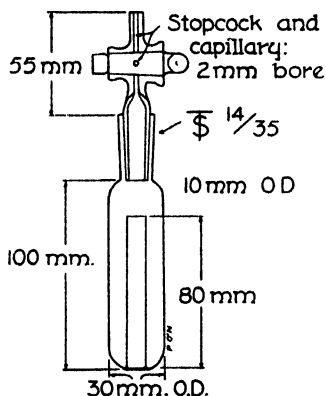


FIG. 1. Modified apparatus C of Van Slyke *et al.* (8) equipped with internal well

equipped with an internal well having a capacity of 6 to 8 ml. (see Fig. 1).³ It has been found advisable to grind further with care the standard taper stop-cock of the adapter with a very fine carborundum powder in order to obtain a surface which seldom needs to be regreased. Likewise, the standard taper joint of the combustion tube, if reground, seems to maintain a better seal with phosphoric acid when heated under the conditions of the analysis. The dimensions of the stop-cock bore and tubing and the preparation of the rubber connectors should be in accordance with the directions of Van Slyke *et al.* (8). In addition, however, the greasing of the stop-cock is of critical importance, as traces of ordinary stop-cock grease may yield catastrophic amounts of CO_2 if contact is made with hot bichromate-sulfuric acid. We have found a very thin film of Silicone

³ Initially, a side arm was introduced into apparatus C for admission of the fluid analyzed (7). The apparatus described above was later found to be much more convenient.

lubricant (Dow-Corning Corporation, Midland, Michigan) to be satisfactory, as it has only a slight and slow reducing activity toward the bichromate mixture used. Care is taken to avoid accumulation of bits of grease at the edge of the stop-cock bore when the key is seated. Before an analysis is started, the apparatus containing some of the oxidizing mixture is heated for a few minutes. The position of the tube on a steam bath is adjusted so that the oxidant makes contact with the stop-cock key. Subsequent analyses serve to keep the apparatus continually clean until the stop-cock needs regreasing.

2. Distillation apparatus with vertical condenser, as described by Harger (10), and equipped with receiving tubes calibrated to contain 25 ml.

Reagents—

1. Bichromate-sulfuric acid oxidizing mixture. 6.6 gm. of potassium bichromate, c.p., are dissolved in a minimum amount of distilled water and made up to 1000 ml. with concentrated sulfuric acid, c.p.

2. Reagents for the determination of CO_2 , as described by Van Slyke *et al.* (8) (0.5 N NaOH, CO_2 -free (hydrazine omitted); 2 N lactic acid; 5 N NaOH, CO_2 -free). It is convenient to prepare the 0.5 N NaOH so that it is highly colored with brom thymol blue or alizarin, thus affording later certainty that the fluid in the manometric chamber is acid when CO_2 is liberated and alkaline after CO_2 absorption.

3. Phosphoric acid, c.p., concentrated (85 per cent).

4. Reagents for the preparation of Folin-Wu tungstate filtrates of whole blood (10 per cent sodium tungstate, 0.667 N H_2SO_4).

5. CO_2 -free acidulated water. To 250 ml. of distilled water add 1 drop of strong sulfuric acid and boil briefly.

Procedure

Preparation of Distillates—Prepare a tungstate filtrate (1:10) from 3 ml. of blood (or serum or plasma) and transfer 20 ml. of the filtrate to the flask of the distilling apparatus. Add 1 or 2 ml. of the CO_2 -free acidulated water to the receiving tube and place it so that the tip of the condenser is beneath the surface of the acidulated water. Surround the lower half of the receiving tube with an ice bath. Distil rather rapidly until about 15 ml. of the distillate have been obtained. Lower the receiving tube so that the tip of the condenser is above the level of the distillate, disconnect the distilling flask, and wash the lumen of the condenser and its tip with a fine spray of CO_2 -free acidulated water. Bring the contents of the receiving tube to room temperature and make up to 25 ml. with the CO_2 -free acidulated water. Stopper the tube, mix the contents, and store in the ice box unless the rest of the analysis is to be conducted at once. Similarly distil a "filtrate," in which water has been substituted for blood, for

subsequent use as a blank. 5 ml. aliquots of the distillates are employed in the oxidation.

Combustion of Alcohols—Introduce 5 ml. of a distillate into the internal well of the scrupulously clean (see above) combustion tube, taking care to avoid contamination of the sample with CO_2 from breath or entry of the latter into the tube. Transfer 5 ml. of the bichromate-sulfuric acid mixture to the external chamber of the tube without wetting the walls of the joint. Connect the adapter and tube by using a small amount of phosphoric acid as a joint seal. Place the tube in an ice bath (stop-cock closed) for about 5 minutes, or until the fluid contents are chilled. Connect the apparatus with a piece of tubing leading to the water pump and evacuate for several seconds. Disconnect, allowing room air to enter the apparatus. Repeat this twice and allow 15 seconds for the third evacuation, this time closing the stop-cock before disconnecting the apparatus from the pump. (Loss of alcohol from the small surface of the liquid in the internal well seems negligible during this operation, and, if the fluid is cold, bumping will not occur during evacuation.) Now gently mix the fluids in the tube. The initial violent reaction immediately subsides. Shake the tube to insure complete mixing and place it in the steam bath, supported so that the joint and stop-cock are not exposed directly to the steam or hot water. Heat for 10 minutes, cool the tube under the tap just enough that it may be handled, and attach it to the side arm leading to the chamber of the Van Slyke-Neill analyzer, which previously has been prepared as described below. The combustion tube should be almost horizontal and it is convenient to suspend the apparatus in such a way that it need not be supported by hand subsequently.

Absorption of CO_2 —Prior to attaching the combustion tube, the chamber of the manometric apparatus is cleaned and degassed by shaking 2 to 3 ml. of 2 N lactic acid at reduced pressure. After expelling the acid, mercury is run into both capillaries from cock *b*.⁴ 0.5 N NaOH, CO_2 -free, is admitted exactly to the 2 ml. mark, and the cock is closed and sealed with mercury.⁵ The mercury level is then lowered to nearly the 50 ml. mark, cock *a* closed, and the leveling bulb is placed in the lower position. Cock *b* and the stop-cock of the combustion tube are now opened so that connection is made with the manometric chamber. Cock *a* is opened and the rise of the mercury level is controlled with the leveling bulb. The CO_2 is now transferred and absorbed by alternately raising and lowering the level

⁴ See Fig. 3 (8).

⁵ 1 drop of 1:4 caprylic acid-ethyl alcohol may be used if foaming is encountered. Slightly more than 2 ml. of alkali are admitted into the chamber by the procedure described. The volume increment is the same in both the blank and unknown and the effect on the value of the factor converting $p\text{CO}_2$ to mg. of C or CO_2 is negligible.

of the mercury fifteen times from the 50 ml. mark to the upper shoulder of the manometric chamber, either by hand or by employing the automatic device for this purpose devised by Van Slyke and Folch (9). During absorption the combustion tube is agitated by hand, with care not to splash the oxidizing mixture so that it enters the manometric chamber. The pressure during absorption should be 50 mm. of Hg or less with the mercury at the 50 ml. mark. Upon completion of absorption close cock *a* with the mercury at the 50 ml. mark in the chamber. Close cock *b* and detach the combustion tube. Seal the capillary leading from the combustion tube to the manometric chamber with mercury, and then expel the gas in the chamber without loss of fluid so that the level of the alkali comes just to the base of the cup. Fill the cup exactly to the 1 ml. mark with 2 N lactic acid and admit all of this to the chamber. Seal with mercury, lower the mercury level to the 50 ml. mark, and shake for 2 minutes. The fluid in the chamber should be acidic. Read the pressure of CO_2 at the 2.0 ml. mark (p_1). Add the number of drops of 5 N alkali to the cup, which previously has been determined to be required to alkalize the solution in the chamber, and admit all of this, taking care to avoid admission of any air bubbles. Seal with mercury. Allow the chamber contents to rise to the top of the pipette to dilute the 5 N alkali on the walls of the pipette and then lower the fluid level to the upper shoulder of the chamber. After allowing 15 seconds for drainage, raise the fluid level to the 2.0 ml. mark and again read the pressure (p_2). Expel the contents of the chamber, wash, and degas with 2 N lactic acid to prepare the chamber for a subsequent analysis.

The blank is determined in exactly the same manner as that described above, and in our experience amounts to about 20 mm. of Hg.

Calculations

The methyl alcohol content is calculated as follows:

$$(p_1 - p_2)_{\text{unknown}} - (p_1 - p_2)_{\text{blank}} = p\text{CO}_2$$

$p\text{CO}_2 \times f = \text{mg. of C as CO}_2 \text{ obtained from the distillate analyzed.}$ *f* for the various temperatures is obtained from the table of factors for the conditions described above which are given by Van Slyke *et al.* (8). When 5 ml. of the distillate are oxidized, $\text{mg. of C} \times 667 = \text{mg. of methyl alcohol per 100 ml. of blood.}$

Results

A series of standard solutions was prepared by diluting absolute methyl alcohol (sp. gr. 0.796 at 15°) with boiled, acidulated distilled water. These solutions ranged from 1.6 to 24 mg. per cent final concentration, and 5 ml.

aliquots corresponded to distillates from bloods containing 20 to 300 mg. per cent of methyl alcohol. Analyses of these standards, in which the distillation process was omitted, gave recoveries of 98.2 to 101.3 per cent (Table I). Similar recoveries were obtained when the same standards were prepared to contain ethyl alcohol also (Table I).

TABLE I

Recovery of Methyl Alcohol in Analyses of Standard Solutions with and without Added Ethyl Alcohol

Methyl alcohol standard mg. per cent	Recovery of methyl alcohol		Methyl alcohol standard mg. per cent	Ethyl alcohol mg. per cent	Recovery of methyl alcohol	
	mg. per cent	per cent			mg. per cent	per cent
1.60	1.57	98.2	1.60	4	1.58	98.8
3.20	3.24	101.3	3.20	4	3.15	98.5
6.40	6.41	100.2	6.40	8	6.37	99.5
9.60	9.61	100.1	9.60	16	9.67	100.6
16.00	15.96	99.7	9.60	40	9.63	100.4
19.20	19.23	101.3	16.00	40	16.06	100.1
24.00	23.84	99.3	24.00	16	24.12	100.4

TABLE II

Recovery of Methyl Alcohol Added to Blood with and without Addition of Ethyl Alcohol

Methyl alcohol in specimen mg. per cent	Recovery		Ethyl alcohol present mg. per cent	Recovery	
	mg. per cent	per cent		mg. per cent	per cent
20	19.6	98.0	50	19.7	98.4
40	39.3	98.2	100	40.6	101.4
80	80.4	100.4	200	79.2	99.0
120	122.3	102.0	500	118.8	98.9
200	198.8	99.4	500	201.5	100.6
240	237.3	98.9	200	238.0	99.2
300	302.5	100.8	500	297.1	98.9

Two series of standard blood samples were prepared. To one series, graded amounts of a standard solution of methyl alcohol were added to produce final blood concentrations ranging from 20 to 300 mg. per cent. To the blood of the other series various amounts of ethyl alcohol, in addition to the methyl alcohol, were also added. The blood was immediately precipitated and distilled, and the distillates analyzed as described. Recoveries of the added methyl alcohol ranged from 98 to 101 per cent, independently of the concentration of ethyl alcohol in the specimen (Table II).

DISCUSSION

Considerable difficulty was experienced at first in attaining the recoveries reported in blood containing less than 50 mg. per cent of methyl alcohol. In these instances the blank $p\text{CO}_2$ is a considerable fraction of the $p\text{CO}_2$ of the sample analyzed and meticulous care is necessary to obtain blanks varying by less than 1 mm. of $p\text{CO}_2$. At higher concentrations, blank variations of 2 to 3 mm. introduce comparatively little error into the calculations. In applying the method in forensic work it has been found convenient to estimate roughly the amount of methyl alcohol present by a qualitative test (13), and in those instances in which the concentrations appear to be less than about 50 mg. per cent, 30 ml. of the tungstate filtrate are distilled. We have not been able to distil quantitatively the alcohol from larger volumes of filtrate without increasing the final volume in the receiving tube beyond 25 ml.

Bumping of the distillate in the well during evacuation is quite apt to occur unless the liquid is effectively chilled. This chilling may be greatly facilitated by placing the combustion apparatus in an ice bath for several minutes before the sample is added to the well. When this is done, less than 5 minutes of subsequent chilling will invariably suffice.

SUMMARY

A manometric method for determining methyl alcohol in blood has been described. Carbon dioxide, liberated from methyl alcohol by bichromate oxidation of a blood distillate in a special combustion chamber, is transferred to and measured in the Van Slyke-Neill apparatus. The procedure is independent of standardized solutions. The presence of ethyl alcohol does not interfere with the determination of methyl alcohol.

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METABOLISM OF C¹⁴-LABELED GLYCINE, L-HISTIDINE, L-LEUCINE, AND L-LYSINE*

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A study of the rates of incorporation *in vivo* of labeled amino acids into tissue proteins was undertaken to find the best time at which intermediates in the process might be found. The rates observed were so fast that we decided to investigate the process in some detail. The amino acids were L- α -aminoadipic-6-C¹⁴ acid, glycine-1-C¹⁴, L-histidine-2-C¹⁴-imidazole, L-leucine-1-C¹⁴, and L-lysine-1-C¹⁴.

There have been no studies in which the metabolism of intravenously injected carbon-labeled amino acids has been followed at short time intervals. Olsen *et al.* (1) fed C¹³-labeled glycine to mice and 16 hours later found questionable amounts of the labeled carbon in the proteins, presumably because the glycine fed contained too little C¹³. With C¹⁴-labeled amino acids there has been no difficulty in finding their incorporation into tissue proteins (2-6). With the exception of the study by Greenberg and Winnick (3) the shortest time interval at which observations were made after the injection or feeding of an amino acid was 6 hours. Greenberg and Winnick made their first set of observations $\frac{1}{4}$ hour after injection of C¹⁴-labeled glycine and their next 6 hours later. The observations reported below indicate that at such time intervals the rapidity of the incorporation into the visceral and serum proteins is missed.

Labeled Amino Acids

Syntheses of the following amino acids have been described: L- α -aminoadipic-6-C¹⁴ acid (7), glycine-1-C¹⁴ (8), L-leucine-1-C¹⁴ (9), L-lysine-1-C¹⁴ (9).

The synthesis of L-histidine-2-C¹⁴-imidazole was a modification of Tesar and Rittenberg's (10) synthesis of L-histidine labeled with N¹⁵, which in turn was a modification of Ashley and Harington's procedure (11). NaSC¹⁴N is condensed with α , δ -diamino- γ -ketovaleric acid dihydrochloride.

* This work is a part of that done under contract with and joint sponsorship of the United States Atomic Energy Commission and the Office of Naval Research, United States Navy Department. The C¹⁴ used in this investigation was supplied by the Carbide and Carbon Chemicals Corporation, Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.

ride to give thiol-L-histidine, which on oxidation with ferric sulfate gives histidine. The amino acid was purified via the diflavianate.

NaSC¹⁴N was prepared by the following adaptation of the method of Castiglioni (12). 56 mg. of NaC¹⁴N and 40 mg. of sulfur were refluxed with 1.7 ml. of acetone for 45 minutes. The acetone was then drawn off and the insoluble residue refluxed again with 20 mg. of sulfur in 1.5 ml. of acetone for 45 minutes. The combined acetone solutions were evaporated in a 3 ml. centrifuge tube with a stream of nitrogen. The residue, which consisted of NaSC¹⁴N and some sulfur, was dried over H₂SO₄. Titration of the thiocyanate in similar test runs showed that the NaC¹⁴N is converted quantitatively to NaSC¹⁴N.

With four 0.5 ml. portions of water (centrifuging each time to remove the sulfur) the NaSC¹⁴N was transferred to a flask containing 835 mg. of α , δ -diamino- γ -ketovaleric acid dihydrochloride and 296 mg. of non-isotopic KSCN. The mixture was heated on a steam bath for 1 hour. Then it was poured dropwise and with constant mixing into a 10 per cent solution of HgSO₄ in 5 per cent H₂SO₄ and the mixture allowed to stand 20 hours at room temperature. The precipitate was collected, washed three times with water, suspended in 150 ml. of water, and decomposed with H₂S. The filtrate from the HgS was heated to remove H₂S and then brought to pH 8 with Ba(OH)₂. The BaSO₄ was filtered and the thiol-histidine solution concentrated *in vacuo* to about 2 ml.

The concentrate was heated on a steam bath for 1 hour after the addition of 8 gm. of ferric sulfate and 40 ml. of water. 200 ml. of hot water were added and, while hot, an excess of Ba(OH)₂. The precipitate was filtered and extracted with boiling water, and the combined filtrates were brought to pH 4.0 with H₂SO₄. After the BaSO₄ was removed, the solution was concentrated to 5 ml. and 550 mg. of flavianic acid were dissolved in it with heating. The diflavianate was allowed to separate first at room temperature, then at 0° overnight. It was washed twice with absolute ethanol and twice with ether. M.p., 240–245°; yield, 486 mg.

484 mg. of radioactive and 760 mg. of non-radioactive L-histidine diflavianate were dissolved in 12 ml. of 2 N H₂SO₄ and extracted seven times with warm *n*-butanol. The aqueous phase was boiled with norit, filtered, and brought to pH 7.1 with Ba(OH)₂. The filtrate from the BaSO₄ was concentrated *in vacuo*, transferred to a small centrifuge tube, and dried in a stream of nitrogen. The crystalline L-histidine was dissolved in 0.5 ml. of hot water and allowed to cool slowly to 0°; 2 to 3 volumes of absolute ethanol were added after the bulk of the crystals had separated. A main crop of 210 mg. of L-histidine-2-C¹⁴-imidazole was obtained after washing three times with absolute ethanol and once with ether. M.p., 272° (decomposition).

Procedure

Our first experiments were carried out on guinea pigs and rabbits, and some of the results obtained with them are presented. In most of the experiments mice were used in order to conserve labeled amino acids. A single experiment consisted of a group of six mice. The labeled amino acid was injected into the tail vein and the animals were killed singly 10, 20, 30, 60, 120, and 240 minutes later.

Radioactivity measurements were made on the expired CO_2 , on the proteins of the blood and viscera, on the CO_2 evolved in ninhydrin treatment of blood and non-protein filtrates before and after complete acid hydrolysis, on the CO_2 from the wet combustion of whole blood and from the wet combustion of the non-protein filtrate of blood and viscera.

The blood was caught, after the animal was killed by a blow on the head, in a 20 ml. beaker containing 0.02 ml. of heparin (1000 units per ml.). A measured volume of the blood was pipetted into a 25 ml. volumetric flask containing 0.01 N NaOH and made up to the mark with the latter solution. One aliquot was taken for precipitation of the proteins; on another 2 ml. aliquot a wet combustion was carried out with the oxidizing mixture of Van Slyke and Folch (13), the oxidation chamber and trap assembly of McCready and Hassid (14), and the CO_2 absorption tower of Weinhouse (15); a 10 ml. aliquot was treated with ninhydrin for the determination of free amino acids by the method of Van Slyke *et al.* (16). The combined thoracic and abdominal viscera were weighed, minced, boiled for 15 minutes in 20 ml. of water, and homogenized; the suspension was brought to pH 5, boiled again for 15 minutes, and filtered; the coagulum was extracted exhaustively with boiling water and the combined filtrate and washings were made up to 100 ml. with water. The coagulum was dried at 100° and weighed. A 2 ml. aliquot of the non-protein filtrate was taken for wet combustion, a 20 ml. aliquot for determination of radioactive free amino acids by the ninhydrin method, and a 10 ml. aliquot was hydrolyzed for 20 hours with 20 per cent HCl; after removal of the excess HCl by distillation, the total radioactive amino acids were measured by the ninhydrin method. The increase in radioactivity in the CO_2 evolved by ninhydrin after acid hydrolysis was taken as a measure of labeled amino acid in combination other than with protein.

In all of the experiments with mice the carcass, after bleeding and removal of the viscera, was dropped into 100 ml. of 30 per cent KOH, allowed to stand at room temperature until the carcass was dissolved, then refluxed at a bath temperature of 150° overnight, cooled, and made up to 150 ml. with water. A 2 ml. aliquot was taken for wet combustion. In

test runs we recovered only 50 to 75 per cent of the radioactivity of added amino acids. We have not investigated at what point the losses occurred. One of the difficulties was that the specific activity of the resulting BaCO₃ was so low. The results obtained are considered as indicating roughly only whether there was a large or small fraction or practically none of the injected radioactivity in the carcass.

7 per cent trichloroacetic acid was used to precipitate the blood proteins and to suspend a small portion of the heat-coagulated visceral proteins. After standing overnight at room temperature, the proteins were washed, dried, and their radioactivity was measured by the methods previously described (17).

TABLE I

Metabolism of Glycine-1-C¹⁴ in Mice

Distribution of radioactivity as per cent of quantity injected.

Weight of animal	Time after injection	Blood			Viscera					Expired CO ₂ , cumulative	Total in blood, viscera, and expired CO ₂ , cumulative
		Total	Protein	Non-protein fraction	Protein	Non-protein fraction			Bound		
						Total	Liberated by ninhydrin reagent				
							Before hydrolysis	After hydrolysis			
<i>gm.</i>											
23	10 min.	1.4	0.1	1.3	6.7	54.2	23.1	26.0	2.9	2.6	64.9
24	20 "	2.1	0.3	1.7	20.9	48.2	19.9	24.0	4.1	9.5	80.7
22	30 "	2.1	2.1	0	34.3	47.3	20.7	25.2	4.5	15.2	98.9
17	1 hr.	2.3	2.3	0	36.8	34.0	6.6	17.6	11.0	22.4	95.5
19	2 hrs.	2.2	2.2	0	45.4	15.1	3.9	7.0	3.1	22.9	85.6
24	4 "	3.2	3.2	0	46.3	10.9	2.1	8.6	6.7	28.4	88.8

The glycine gave 55,000 c.p.m. per mg. Injected 0.7 mg. = 38,500 c.p.m.

Results

Tables I to V summarize the main results of the experiments on mice. The features common to the metabolism of glycine, histidine, leucine, and lysine were the rapid disappearance of the labeled amino acid from the blood (so rapid that 10 minutes after injection less than 3 per cent was in the blood), the appearance of radioactivity in the blood proteins later than in the visceral proteins, the very rapid incorporation of labeled amino acids into the visceral proteins, attaining at least 75 per cent of the maximum $\frac{1}{2}$ hour after injection, the more rapid decrease of labeled amino acid in the non-protein fraction of the viscera than in their proteins, the small fraction of bound labeled amino acids, and the rapid appearance of

labeled CO₂ in the expired air, attaining nearly its maximum 1 hour after injection.

TABLE II
Metabolism of L-Histidine-2-C¹⁴-Imidazole in Mice
Distribution of radioactivity as per cent of quantity injected.

Weight of animal	Time after injection	Blood			Viscera		Expired CO ₂ , cumulative	Total in blood, viscera, and expired CO ₂ , cumulative
		Total	Protein	Non-protein fraction	Protein	Non-protein fraction*		
gm.								
24	10 min.	2.3	0.1	2.2	8.5	29.8	1.3	41.9
25	20 "	2.6	0.8	1.8	17.8	20.3	9.3	50.0
24	30 "	2.3	1.6	0.7	40.8	10.5	26.1	79.7
26	1 hr.	3.5	2.6	0.9	45.4	6.5	35.6	91.0
24	2 hrs.	4.8	4.8	0	40.4	6.2	39.8	91.2
22	4 "	4.9	4.9	0	38.3	5.8	43.3	92.3

* Ninhydrin determinations were carried out on the non-protein filtrate before and after hydrolysis. The liberated CO₂ gave no counts.

The histidine gave 35,900 c.p.m. per mg. Injected 1.47 mg. = 52,800 c.p.m.

TABLE III
Metabolism of L-Leucine-1-C¹⁴ in Mice
Distribution of radioactivity as per cent of quantity injected.

Weight of animal	Time after injection	Blood			Viscera					Expired CO ₂ , cumulative	Total in blood, viscera, and expired CO ₂ , cumulative
		Total	Protein	Non-protein fraction	Protein	Non-protein fraction			Bound		
						Total	Liberated by ninhydrin reagent				
							Before hydrolysis	After hydrolysis			
gm.											
28	10 min.	1.9	0.2	1.7	16.7	7.2	7.1	7.1	0	3.3	28.9
19	20 "	1.6	0.1	1.5	26.1	12.9	5.0	5.2	0.2	18.6	59.2
24	30 "	1.0	0.7	0.3	46.8	5.2	2.3	3.4	1.1	39.8	92.8
24	1 hr.	2.0	2.0	0	33.3	3.4	0.8	1.1	0.3	58.3	97.0
23	2 hrs.	2.8	2.8	0	35.6	5.2	1.9	2.2	0.3	54.2	97.8
22	4 "	3.7	3.7	0	38.2	7.0	2.8	2.9	0.1	56.8	105.7

The leucine gave 15,000 c.p.m. per mg. Injected 2.0 mg. = 30,000 c.p.m.

Analysis of the carcass indicated the presence of a large fraction of the injected amino acid in the first 10 to 20 minutes after the injection, after which time it rapidly decreased; therefore after 1 hour little or no radioactivity could be detected.

Table VI shows more clearly than Tables I to IV the initially more rapid incorporation of labeled amino acids into the visceral than into the

TABLE IV
Metabolism of L-Lysine-1-C¹⁴ in Mice

Distribution of radioactivity as per cent of quantity injected.

Weight of animal	Time after injection	Blood			Viscera						Expired CO ₂ , cumulative	Total in blood, viscera, and expired CO ₂ , cumulative
		Total	Protein	Non-protein fraction	Protein	Non-protein fraction				Bound		
						Total	Liberated by ninhydrin reagent					
							Before hydrolysis	After hydrolysis				
gm.												
25	10 min.	2.2	0	2.2	20.0	20.9	17.1	17.1	0	2.7	45.8	
29.5	20 "	3.5	0.1	3.4	32.8	11.3	9.1	9.5	0.4	8.8	56.4	
27	30 "	1.0	0.2	0.8	17.9	7.8	3.8	5.8	2.0	14.3	41.0	
20	1 hr.	0.6	0.2	0.4	19.4	6.9	2.2	3.8	1.6	21.4	48.3	
23	2 hrs.	2.7	0.8	1.5	24.0	6.0	2.7	2.9	0.2	25.6	58.3	
26.5	4 "	2.8	0.8	1.6	23.0	4.4	1.7	2.3	0.6	27.5	57.7	

The lysine gave 19,500 c.p.m. per mg. Injected 1.46 mg. = 28,500 c.p.m.

TABLE V
Metabolism of L- α -Aminoadipic-6-C¹⁴ Acid in Mice

Distribution of radioactivity as per cent of quantity injected.

Weight of animal	Time after injection	Blood			Viscera		Expired CO ₂ , cumulative	Total in blood, viscera, and expired CO ₂ , cumulative
		Total	Protein	Non-protein fraction	Protein	Non-protein fraction*		
22	10 min.	0.6	0	0.6	0	6.6	1.2	8.4
	20 "						6.0	
25	30 "	0.5	0	0.5	0	19.7	14.9	35.1
26	1 hr.	0.07	0	0.07	0	12.5	31.5	44.1
20	2 hrs.						46.6	
	4 "	0	0	0	0	2.4	55.3	57.7

* Ninhydrin determinations were carried out on the non-protein filtrate before and after hydrolysis. The liberated CO₂ gave no counts.

The α -aminoadipic acid gave 14,450 c.p.m. per mg. Injected 1.2 mg. = 17,350 c.p.m.

plasma proteins, followed by an approach to a balance between them. In every case the ratio of the specific activity of the visceral to that of the plasma proteins was high at first, greatly in excess of 1, and later, after 1 to 2 hours, approached a value near 1.

The specific activity of the plasma proteins was not measured directly; all of the proteins of the blood were precipitated with trichloroacetic acid. It was assumed that none of the labeled amino acids had been incorporated into the hemoglobin and that all the radioactivity in the precipitate of mixed proteins was in the plasma proteins. The total blood in the animal and the fraction, as plasma proteins, was calculated as follows: weight of blood = 6.4 per cent of body weight (18), specific gravity of blood = 1.06; the blood contained 23 per cent of proteins, of which 30 per cent was

TABLE VI

Incorporation in Mice of C¹⁴-Labeled Amino Acids into Proteins of Viscera and of Blood at Different Time Intervals

Time after injection	Specific activity of proteins of			Visceral protein Plasma protein calculated	Time after injection	Specific activity of proteins of			Visceral protein Plasma protein calculated
	Viscera	Blood	Plasma, calcu- lated			Viscera	Blood	Plasma, calcu- lated	
*Counts per min. per mg. protein					Counts per min. per mg. protein				
Glycine					L-Leucine				
10 min.	2.2	0.1	0.3	7.3	10 min.	2.7	0.2	0.7	3.8
20 "	6.9	0.4	1.3	4.6	20 "	5.9	0.1	0.3	19.6
30 "	10.6	1.5	5.0	2.1	30 "	7.1	0.8	2.6	2.7
1 hr.	10.7	1.7	5.6	1.9	1 hr.	7.1	2.5	8.3	0.85
2 hrs.	9.3	2.1	6.9	1.3	2 hrs.	7.9	2.5	8.3	0.95
4 "	9.6	2.6	8.6	1.1	4 "	8.5	3.5	11.6	0.73
L-Histidine					L-Lysine				
10 min.	3.6	0.2	0.7	5.1	10 min.	3.3	0	0	
20 "	7.3	1.1	3.6	2.0	20 "	4.9	0.07	0.02	24.5
30 "	16.2	2.6	8.6	1.9	30 "	4.7	0.2	0.7	6.7
1 hr.	17.5	5.2	17.2	1.0	1 hr.	5.5	1.0	3.3	1.7
2 hrs.	16.1	7.6	25.1	0.64	2 hrs.	5.9	1.1	3.6	1.6
4 "	17.6	8.3	27.4	0.64	4 "	5.2	0.9	3.0	1.7

plasma proteins. The specific activity of the plasma proteins was, then, taken as $3.3 \times$ the observed specific activity of the total blood protein.

Although each of the experiments of Tables I to VI was carried out twice and gave the general metabolic picture described above, we draw no specific conclusions from the quantitative differences found among the rates of metabolism of the amino acids except that of α -amino adipic acid. This amino acid was different from the other four in that, although the L form was injected, none was incorporated into the visceral or plasma proteins. It was oxidized to yield its C¹⁴ in the expired air approximately as fast as was lysine, if we take into account that there was less of the

lysine available for oxidation since 20 per cent had been incorporated into the visceral proteins. Our findings with α -aminoadipic acid are in accord with that of Miller and Bale (19) who fed DL-lysine-6-C¹⁴ to a dog and found 24 hours later no evidence of α -aminoadipic acid in the proteins. It appears, therefore, that α -aminoadipic acid is a catabolic product of lysine in animal tissues (7, 20) and is not a normal constituent of animal proteins.

In order to compare the rates of incorporation of the labeled amino acids observed in mice with those in other animals and under other conditions, the rates of incorporation into the visceral proteins are summarized in Table VII in terms of micromoles of amino acid incorporated per gm. of protein (dry weight) per hour. The data show that the incorporation

TABLE VII

Amounts of Labeled Amino Acids Found in Visceral Proteins of Mice at Successive Time Intervals after Intravenous Injection

The amounts are expressed as rates per hour in the interval.

Time	Glycine	Histidine	Leucine	Lysine
	μM per gm. protein per hr.			
<i>min.</i>				
10	3.2	3.4	8.2	6.9
20	4.3	3.9	9.0	5.1
30	5.2	5.8	7.2	3.4
60	2.5	3.1	3.6	1.9
120	1.1	1.4	2.0	1.0
240	0.6	0.8	1.0	0.4

of glycine and of histidine proceeded unslackened for 30 minutes after the injection; with leucine and lysine the maximum rates came earlier. 30 minutes after injection the rates diminished progressively, until after 4 hours there was practically no further increase in labeled amino acids in the proteins.

The rates of incorporation were similar in rabbits 30 minutes after intravenous injection (40 mg. per kilo) of leucine. Expressed in Table VII as micromoles per gm. of protein per hour, representative rates were as follows: in heart 2.4, small intestine 6.6, kidney 4.0, liver 5.6, skeletal muscle 1.8, red blood corpuscles (washed) 0.26, serum 1.0, and spleen 5.4.

Somewhat slower rates were found in guinea pigs 30 minutes after the intravenous injection of 16 mg. per kilo of glycine, histidine, leucine, and lysine; the rates found for glycine, histidine, leucine, and lysine, respectively, in small intestine were 1.3, 0.90, 3.5, and 2.3, and in liver 0.80, 1.7, 2.3, and 2.0 μM per gm. of protein per hour.

We have reported (9) rates of incorporation *in vitro* in different fractions of guinea pig liver homogenate. It was interesting to compare these rates with those *in vivo* 30 minutes after injection of 16 mg. per kilo of the amino acid. The units are the same as above; the values in parentheses are those previously observed *in vitro*: glycine, "nuclear" fraction 0.56 (0.52); mitochondria 0.6 (0.40); microsomes 1.2 (0.075); supernatant 0.69 (0); histidine "nuclear" fraction 1.5 (0.32);¹ mitochondria 1.2 (0.15);¹ microsomes 3.1 (1.5);¹ supernatant 1.2 (3.7);¹ leucine, "nuclear" fraction 2.3 (0.61); mitochondria 1.1 (?); microsomes 4.3 (0); supernatant 1.8 (0); lysine, "nuclear" fraction 1.3 (4.1); mitochondria 1.6 (3.2); microsomes 2.9 (0.9); supernatant 1.6 (3.2). *In vivo* the greatest incorporation of every amino acid was in the microsome fraction and it was much greater than that observed in that fraction *in vitro*. The one other consistent difference between the findings *in vivo* and *in vitro* was that there was considerable incorporation *in vivo* of each of the amino acids in every fraction. *In vitro* there was little or no incorporation of glycine and leucine in the microsome and supernatant fractions.

SUMMARY

1. The synthesis of L-histidine-2-C¹⁴-imidazole is described.
2. C¹⁴-Labeled L- α -amino adipic acid, glycine, L-histidine, L-leucine, and L-lysine were injected intravenously into mice. Within less than 10 minutes nearly all of the amino acid had disappeared from the blood into the carcass. Within the next 30 minutes 18 to 47 per cent of the injected amino acid (except α -amino adipic acid) was incorporated into the visceral proteins; after an hour both the incorporation into the visceral proteins and the initial burst of oxidation of the amino acid had attained nearly their maxima. 1 hour after the injection onward a significant amount of labeled amino acid appeared in the plasma proteins, and within 2 hours the visceral and plasma proteins were in balance with respect to labeled amino acid content.
3. No α -amino adipic acid was incorporated into either the visceral or serum proteins. It was oxidized at approximately the same rate as lysine.
4. Similarly rapid rates of incorporation were found in the guinea pig and rabbit.
5. Rates of incorporation are given of the labeled amino acids *in vivo* into the proteins of different fractions of guinea pig liver.

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¹ The details of these determinations will be reported in a later communication.

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BIOSYNTHESIS OF DICARBOXYLIC ACIDS BY CARBON DIOXIDE FIXATION

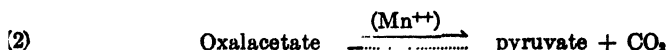
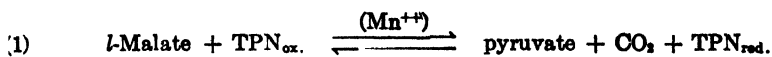
II. FURTHER STUDY OF THE PROPERTIES OF THE "MALIC" ENZYME OF PIGEON LIVER*

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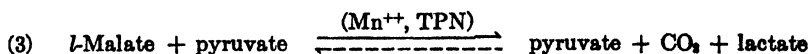
(Received for publication, April 5, 1950)

The isolation, partial purification, and some of the properties of an enzyme from pigeon liver which catalyzes Reactions 1 and 2 have already been described (1).



This enzyme is provisionally referred to as "malic" enzyme. The present paper deals with further observations on the kinetics and other properties of the purified enzyme.¹

The enzyme is totally inactive in the absence of Mn^{++} . Mg^{++} can replace Mn^{++} but is less effective. Further evidence has been obtained indicating that free oxalacetate is not an intermediate in Reaction 1 and that both Reactions 1 and 2 are catalyzed by one and the same enzyme. Additional evidence has also been obtained showing that the dismutation between malate and pyruvate (2, 3), represented by Reaction 3, is catalyzed



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¹ Solid line arrows ($\rightleftharpoons$) will be used to indicate ready reversibility of a reaction; one solid and one dash arrow ($\rightleftharpoons$) to indicate a reversible reaction which occurs predominantly in the direction of the solid arrow; and one solid and one dotted arrow ($\rightleftharpoons$) to indicate that, while the reaction is probably reversible, progress in the direction of the dotted arrow has not been demonstrated. TPN = triphosphopyridine nucleotide; DPN = diphosphopyridine nucleotide.

by a combination of "malic" enzyme and lactic dehydrogenase. As would be expected from the dependence of Reaction 1 on TPN, cytochrome *c* is rapidly reduced by *l*-malate in a system containing pigeon liver "malic" enzyme, cytochrome reductase (4), Mn^{++} , and TPN.

Further work on the biosynthesis of malate from pyruvate and CO_2 , with purified pigeon liver "malic" enzyme, is presented in Paper III (5).

Results

Preparation and Purity of Enzyme—The enzyme was prepared as previously described (1). The combined extracts of 600 gm. of acetone powder contained 980,000 units of specific activity about 5. The final preparation contained 49,600 units of specific activity 500. The yield was thus 5 per cent. It has since been found that, by carrying out the entire procedure on smaller batches (100 gm.) of acetone powder, a similar degree of purification is reached with fewer steps and much higher yields. Preparations of specific activity 600 to 700 with 25 to 30 per cent yield have been obtained in this way.² The fraction of specific activity 500 was dialyzed at 0° against 0.01 M phosphate buffer, pH 7.4, and lyophilized. This resulted in a decrease of the specific activity of the enzyme to 350; the activity of the dry powder, which was stored at 0° in a vacuum over calcium chloride, dropped gradually to about 100 over a period of several months. Solutions of this powder were used for the experiments reported in this paper.

Data illustrating the more or less complete removal of several enzymes originally present in the acetone powder extract are shown in Table I. Similar data have already been presented (1), but those of Table I are more complete; they include glutamic dehydrogenase activity, which had not been previously determined, and activity tests of the various contaminating enzymes at each major step in the purification of the "malic" enzyme. The test for "malic" enzyme (Reaction 1) and oxalacetic carboxylase (Reaction 2) activity were carried out as described above Tables II and III respectively. The optical tests for the other enzymes were as previously described (3). As can be seen from the ratio of other enzymes to "malic" enzyme shown in Table I, both glutamic and isocitric dehydrogenases were completely removed and malic dehydrogenase to a considerable extent. However, the removal of lactic dehydrogenase was still incomplete. We should like to emphasize again the remarkable constancy of the ratio of oxalacetic carboxylase to "malic" enzyme activity (O/E ratio, Table I) throughout purification. This ratio is higher than that previously reported (1) because at pH 4.5, at which the oxalacetic carboxylase tests are now being carried out, the activity of the enzyme is about 20 per cent higher

² I. Harary, J. B. Veiga Salles, and S. Ochoa, unpublished results.

than at pH 5.0. In the more recent preparations referred to above,³ of specific activity 700, the O/E ratio was also 1.2; i.e., it remained constant over a 140-fold purification. These preparations also showed a more complete removal of malic and lactic dehydrogenase, the ratio of malic dehydrogenase to "malic" enzyme averaging 0.15 and that of the lactic dehydrogenase to "malic" enzyme averaging 0.6.

The parallel loss of "malic" enzyme and oxalacetic carboxylase activity

TABLE I

Activity of Various Enzymes at Different Purification Stages of Pigeon Liver "Malic" Enzyme

The figures in parentheses represent the number of tests averaged.

Fraction	Specific activity						Ratios				
	"Malic" enzyme	Oxalacetic carboxylase*	Lactic dehydrogenase	Malic dehydrogenase	Isocitric dehydrogenase	Glutamic dehydrogenase	O/E	L/E	M/E	I/E	G/E
	(E)	(O)	(L)	(M)	(I)	(G)					
Aqueous extract†	5	6.6	280	312	21	12	1.3	56	62	4	2.4
1st ethanol fractionation	21	23	250	155	46	12	1.1	12	7	2	0.6
Refractionated with ethanol‡	48	66	450	122	65	3	1.4	9	2.5	1.4	0.05
Eluted from alumina gel†	190 (5)	236 (2)	850 (5)	130 (2)	44	0	1.2	4.5	0.7	0.2	0
Refractionated with ethanol and alumina gel‡	350 (5)	435	1780 (2)	133	0	0	1.2	5	0.4	0	0

* Tested in the presence of added TPN.

† No glucose dehydrogenase found at levels tested.

‡ Solution of lyophilized enzyme.

when the purified enzyme is heated reinforces the evidence that both activities reside in the same protein. Thus, a dilute solution of enzyme in 0.004 M phosphate buffer, pH 7.4, lost half of its "malic" and half of its oxalacetic carboxylase activity when heated for 5 minutes at 50°; both activities vanished when heated for the same time at 60°. In both cases the lactic dehydrogenase activity of the preparation remained unchanged.

When tested at their respective pH optima and at approximately the same temperature in the presence of optimum substrate concentrations, the rates of the forward Reactions 1 and 2 (per mg. of enzyme protein) are nearly identical. If the extinction coefficient of TPN_{red.} (6) is taken as ϵ

6.22×10^6 (sq. cm. $\times$ mole⁻¹), 1 enzyme unit, as previously defined (1), corresponds to an early rate of reduction of TPN of 0.0048×10^{-6} mole per minute at 23° under the conditions of our standard test. A "malic" enzyme specific activity of 700 corresponds then to a rate of reduction of TPN, or a rate of evolution of CO₂, of $700 \times 0.0048 \times 10^{-6} = 3.36 \times 10^{-6}$ mole per minute per mg. of protein. The same preparation has an oxalacetic carboxylase specific activity of 840; i.e., it catalyzes the evolution of 84 c.mm. (or 3.75×10^{-6} mole) of CO₂ from oxalacetate per minute per mg. of protein at 25°. The above values correspond to a rate of conversion of about 350 moles of malate or oxalacetate per 100,000 gm. of protein per minute. This relatively low degree of activity may indicate that our preparations of pigeon liver "malic" enzyme are still rather impure.

Effect of pH—The pH-activity curves of Reactions 1 and 2 are shown in Fig. 1. In determining the activity of oxalacetic carboxylase at pH values above 5.1, complete evolution of the CO₂ produced by decarboxylation was insured by tipping acid from the side bulb of the Warburg vessels in the usual manner. Both reactions have sharp pH optima. The optimum for Reaction 1 is at pH 7.5, while that for Reaction 2 is at pH 4.5. It is clear from the curves that at pH 7.5 the enzyme catalyzes Reaction 1 exclusively, whereas at pH 4.5 only Reaction 2 is catalyzed. This shows that free oxalacetate cannot be an intermediate in Reaction 1. This conclusion is further supported by the fact that, whereas malonate strongly inhibits decarboxylation of oxalacetate by the pigeon liver enzyme, it does not significantly inhibit Reaction 1 at similar concentrations (see also Korkes *et al.* (7)). The final proof that free oxalacetate is not an intermediate in Reaction 1 was provided by experiments with C¹⁴O₂ (8). Through Reaction 1, C¹⁴O₂ is readily incorporated in the carboxyl of malate β to the alcoholic group, but little or no isotope is incorporated into oxalacetate if this compound is present along with the components of the reaction.²

Kinetics—Although Reaction 1 is readily reversible (1), its equilibrium position is so far to the right that, under the conditions of the standard optical test, it proceeds until practically all of the TPN is reduced. It has not yet been possible to determine the equilibrium constant because of the slight contamination of the enzyme with lactic dehydrogenase (1). The forward reaction follows roughly first order kinetics with respect to TPN until about 70 to 80 per cent of the nucleotide is reduced; at this point it becomes slower than first order. As is shown in Table II, the first order velocity constant, up to 80 per cent TPN reduction, is proportional to the enzyme concentration over a 5-fold range of enzyme dilutions.

While the spontaneous decarboxylation of oxalacetate is kinetically of first order (9, 10), the enzymatic decarboxylation follows zero order kinetics

as long as the enzyme is saturated with substrate (Table III). This is understandable if, in accordance with the Michaelis-Menten theory, the velocity of enzymatic decarboxylation of oxalacetate is proportional to the concentration of an enzyme-Mn-oxalacetate complex.

Within the range of enzyme concentrations illustrated in Table III the

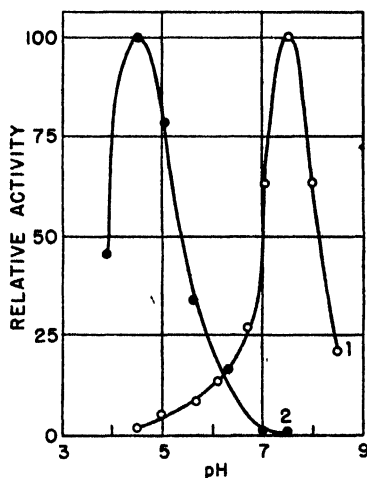


FIG. 1. pH-activity curves of pigeon liver enzyme. Standard tests for "malic" enzyme (Curve 1) and oxalacetic carboxylase (Curve 2) activity except that, in the latter case, $2.5 \mu\text{M}$ of MnCl_2 and $76 \mu\text{M}$ of oxalacetate were present. Final volume, 2.0 cc.; temperature, 23° and 25° respectively. The manometric determination of oxalacetic carboxylase activity at pH values above 5.1 was carried out as described in the text. The buffers used were as follows: pH 3.8 to 5.1, acetate; pH 5.5 to 6.7, citrate; pH 7.0 to 8.5, veronal. At pH 7.5 veronal and glycylglycine buffers gave identical results for "malic" enzyme activity. Final concentration of buffer, 0.025 M in the "malic" enzyme tests and either 0.1 M acetate or citrate or 0.05 M veronal in the oxalacetic carboxylase tests. Freshly prepared solutions of oxalacetic acid were brought to the desired pH with dilute, CO_2 -free sodium hydroxide. The pH values plotted are initial ones. Except in the case of the oxalacetic carboxylase tests, in which the final pH values were about 0.2 unit higher, the actual pH values of the reaction mixtures (as determined at the end with the glass electrode) agreed well with the calculated ones. Lyophilized enzyme; specific activity ("malic" enzyme) at time of use, 325.

concentration of oxalacetate needed to saturate the enzyme has been found to be about 1.4×10^{-3} M. The drop in the rate of CO_2 evolution after 20 and 15 minutes in the samples with 0.081 and 0.121 mg. of enzyme protein, respectively, is due mainly to the oxalacetate concentration falling below the saturation level. Since oxalacetate also undergoes spontaneous decarboxylation, the concentration of the β -keto acid in the experiments of Table III, at the time when the rate of CO_2 evolution dropped, was prob-

ably below 1.3×10^{-2} M. Table III shows that, under the conditions chosen, the rate of CO_2 evolution during the first 15 minutes is proportional to the enzyme concentration over a 7-fold range of enzyme dilutions.

In previous experiments (1) the effect of metallic ions on Reactions 1 and 2 had been studied with relatively crude enzyme fractions of specific activity 100 to 150. Although markedly activated by addition of metal, these preparations were not inactive in its absence. With purer enzyme preparations, addition of Mn^{++} or Mg^{++} is indispensable for activity of both Reaction 1 and 2. Fig. 2 illustrates the dependence of enzyme

TABLE II

Rate of Reduction of TPN by l-Malic Acid As Function of "Malic" Enzyme Concentration

Standard optical test, 0.025 M glycylglycine buffer, pH 7.4, $3 \mu\text{M}$ of MnCl_2 , 0.135 μM of TPN_{ox} , 1.5- μM of l-malate, enzyme, and water. Final volume, 3.0 cc. Corex or silica cells, 1.0 cm. diameter. No TPN in control cell. Reaction started by addition of enzyme. Temperature, 23° . Lyophilized enzyme; specific activity at time of use, 240. Protein values corrected to correspond to initial specific activity of fraction (500) before lyophilization. Initial TPN_{ox} concentration, $c_0 = 0.038 \times 10^{-6}$ mole per cc.

Enzyme concentration	k^*	$\frac{k}{\text{Protein}}$
mg. protein per cc.	min. ⁻¹	
0.0024	0.18	75.0
0.0034	0.22	64.8
0.0050	0.36	72.0
0.0068	0.49	72.0
0.0102	0.68	66.5

* First order velocity constant, $k = (1/t) \ln (c_0/c_t)$, where t = time in minutes, c_0 = initial TPN_{ox} concentration, c_t = TPN_{ox} concentration at time t . Extinction coefficient of TPN_{red} . at 340 $m\mu$ taken as $\epsilon = 6.22 \times 10^6$ (sq. cm. $\times$ mole⁻¹) (6).

activity on the concentration of either Mn^{++} or Mg^{++} . Half maximum rates of Reaction 1 and 2 are reached in each case when the concentration of Mn^{++} is about 5×10^{-5} M. In the case of Mg^{++} the half saturation concentration for Reaction 1 is about 5×10^{-4} M. The curve expressing the dependence of Reaction 2 on the concentration of Mg^{++} is anomalous, owing perhaps to excessive complex formation between Mg^{++} and oxalacetate at the higher concentration of cation required in this case.

The affinity of the pigeon liver "malic" enzyme for l-malic acid is very high. Fig. 3 illustrates the dependence of the rate of Reaction 1 in the forward direction on the concentration of l-malic acid. Half maximum rate is reached when the malate concentration is 5×10^{-5} M. Thus, the

dissociation constant of the enzyme-*l*-malic acid and the enzyme-Mn complex is the same. The spontaneous decarboxylation of oxalacetic acid makes it difficult to obtain an accurate estimate of the half saturation and saturation values of the protein-oxalacetic acid complex in Reaction 2. As stated above, the latter value was found to be in the neighborhood of 1.4×10^{-2} mole of oxalacetate per liter; the half saturation value is about 10^{-3} mole per liter.

TABLE III

Rate of Decarboxylation of Oxalacetic Acid by Pigeon Liver Enzyme As Function of Enzyme Concentration

Standard manometric test, 0.1 M acetate buffer, pH 4.5, $1.0 \mu\text{M}$ of MnCl_2 , $0.033 \mu\text{M}$ of TPN, and $19 \mu\text{M}$ (425 c.mm.) of oxalacetate (1.9×10^{-3} M). Final volume, 1.0 cc. Gas, air; temperature, 25° . Same enzyme as in Table II. Protein values corrected in the same way. Reaction started by tipping in enzyme from side bulb of small Warburg vessels (capacity, about 6 cc.) after temperature equilibration. Measurement of CO_2 evolution started at 5 minutes following addition of enzyme to reaction mixture. CO_2 evolution due to enzyme obtained by subtracting that due to spontaneous decarboxylation of oxalacetate which was measured in parallel runs with neither enzyme nor TPN.

Time	Enzyme concentration, mg. protein per cc.							
	0.0162		0.0405		0.081		0.121	
	CO_2 evolution	$\frac{\text{CO}_2}{t}$	CO_2 evolution	$\frac{\text{CO}_2}{t}$	CO_2 evolution	$\frac{\text{CO}_2}{t}$	CO_2 evolution	$\frac{\text{CO}_2}{t}$
min.	c.mm.		c.mm.		c.mm.		c.mm.	
5	5	1.0	10	2.0	21	4.2	31	6.2
10	10	1.0	22	2.2	43	4.3	61	6.1
15	15	1.0	32	2.13	65	4.3	89	5.92
20	21	1.05	42	2.1	85	4.25	106	5.3
25	26	1.04	51	2.04	98	3.9	120	4.8
CO_2	61.5		52.0		52.5*		50.0†	
$t \times \text{mg. protein}$								

* Calculated for 20 minutes.

† Calculated for 15 minutes.

Inhibitors of Oxalacetate Decarboxylation—It was previously reported (11) that malate strongly inhibits the enzymatic decarboxylation of oxalacetate by crude pigeon liver preparations. This inhibition has now been studied with the purified enzyme; typical results are illustrated in Table IV. It is apparent that *d*-malate is as inhibitory as *l*-malate, whereas, as previously noted, fumarate is practically non-inhibitory. Malonate is a stronger inhibitor than is malate. Evans *et al.* (12) first noticed malo-

nate inhibition of the decarboxylation of oxalacetate by pigeon liver extracts. Later Lwoff *et al.* (13) observed with intact cells of a mutant strain of *Moraxella lwoffii*, that malonate (as well as malate) inhibited the decarboxylation of oxalacetate but not the oxidative decarboxylation of malate, and interpreted this observation to mean that oxalacetate was not an intermediate in the latter reaction. Although malate, malonate, and succinate may be regarded as structural inhibitors of the enzymatic

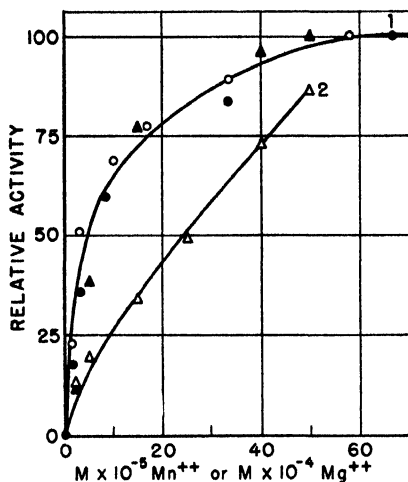


FIG. 2

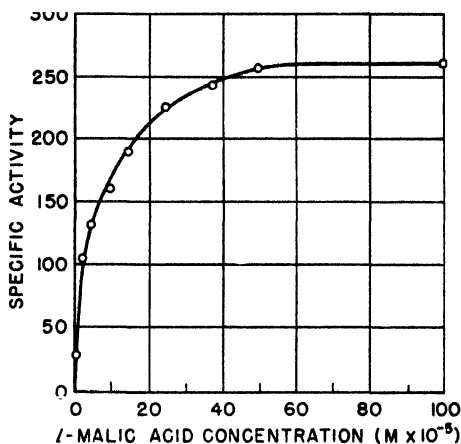


FIG. 3

Fig. 2. "Malic" and oxalacetic carboxylase activities of pigeon liver enzyme as a function of the concentration of Mn^{++} . Standard tests for "malic" enzyme and oxalacetic carboxylase activity. Curve 1, (●) "malic" activity with Mn^{++} , (○) "malic" activity with Mg^{++} , (▲) oxalacetic carboxylase activity with Mn^{++} ; Curve 2, (Δ) oxalacetic carboxylase activity with Mg^{++} . Same enzyme as in Fig. 1.

Fig. 3. "Malic" enzyme activity as a function of the concentration of *l*-malic acid. Standard tests (each with 8 enzyme units). Specific activity of enzyme at time of use, 260.

decarboxylation of oxalacetate, the inhibition seems to be non-competitive, since it is not lessened by increasing the concentration of substrate.

Malate-Pyruvate Dismutation—Evidence has already been presented (3) that the malate-pyruvate dismutation (Reaction 3) is catalyzed by "malic" enzyme together with lactic dehydrogenase, and it was pointed out that this TPN-linked reaction is possible because of the fact that lactic dehydrogenase can react with TPN, although at a much lower rate than with DPN. The earlier experiments with purified pigeon liver "malic" enzyme, carried out at pH 5.2, definitely showed that the amount of lactic dehydrogenase contaminating the preparation was not sufficient for maxi-

TABLE IV

Inhibitors of Decarboxylation of Oxalacetic Acid by Pigeon Liver Enzyme

Conditions as in Table III, except that some experiments were carried out at pH 5.2. Final concentration of oxalacetate, 0.019 M. Enzyme activity (corrected for spontaneous decarboxylation of oxalacetate) calculated for the period between 5 and 10 minutes after tipping in the enzyme. Activity in the absence of inhibitor taken as 100.

Initial pH	Inhibitor	Relative activity	Inhibition
		<i>per cent</i>	<i>per cent</i>
4.5	0.0025 M <i>l</i> -malate	74	26
4.5	0.005 " "	65	35
4.5	0.005 " <i>dl</i> -malate	65	35
5.2	0.001 " <i>l</i> -malate	74	26
5.2	0.005 " "	44	56
5.2	0.01 " "	37	63
5.2	0.01 " <i>dl</i> -malate	37	63
5.2	0.02 " <i>d</i> -malate	35	65
5.2	0.01 " malonate	15	85
5.2	0.01 " succinate	75	25
5.2	0.01 " fumarate	90	10
5.2	0.01 " acetoacetate	88	12
5.2	0.01 " pyruvate	92	8

TABLE V

*Dismutation between *l*-Malate and Pyruvate with "Malic" Enzyme and Lactic Dehydrogenase at pH 7.4*

The complete samples contained 0.05 M glycylglycine buffer, pH 7.4, 0.135 μ M of TPN, 2.5 μ M of MnCl₂, 30 μ M (872 c.mm.) of *l*-malate, 60 μ M of pyruvate, lactic dehydrogenase (6000 units), and "malic" enzyme of specific activity 325 at time of use (245 units in Experiment 1; 225 units in Experiment 2). Final volume, 2.0 cc.; gas, air; temperature, 25°. Reaction started by tipping in "malic" enzyme from the side bulb of Warburg vessels after temperature equilibration. CO₂ evolution determined by tipping in sulfuric acid at the end of the experiment under correction for the CO₂ present initially in control samples.

Sample	CO ₂ evolution in 30 min.	
	Experiment 1	Experiment 2
	<i>c.mm.</i>	<i>c.mm.</i>
Complete	322	
No lactic dehydrogenase	83	
Complete		258
No "malic" enzyme		20
" malate		40
" pyruvate		13
" TPN, 0.15 μ M DPN		10

imum rates of dismutation, since increased rates were obtained on addition of lactic dehydrogenase. However, when it was found that at pH 5.2 the rate of Reaction 1 is but a small fraction of that at pH 7.4, a repetition of the dismutation experiments at the latter pH was desirable. The

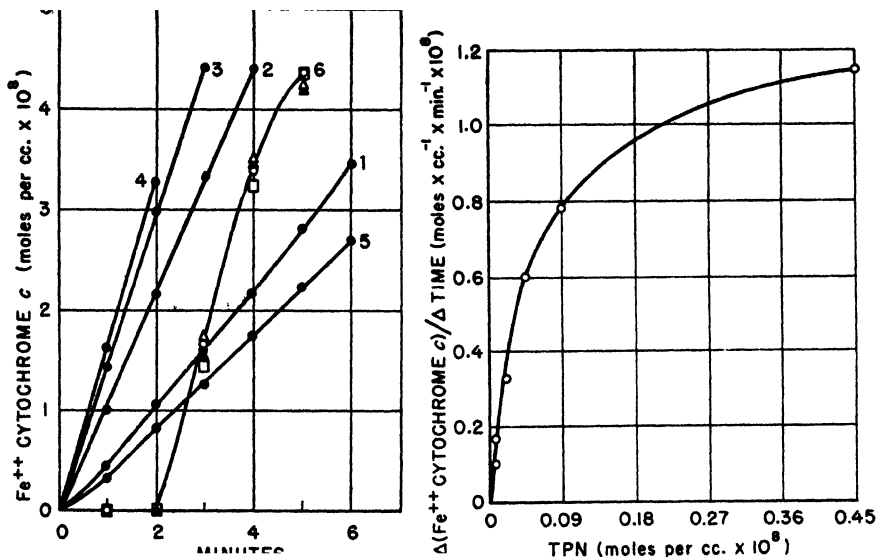


FIG. 5

FIG. 4. Reduction of cytochrome *c* by *l*-malic acid in the presence of "malic" enzyme, TPN, and cytochrome reductase. 0.025 M glycyl-glycine buffer, pH 7.4. 1.5 μ M of MnCl₂, 0.0067 μ M of TPN, 1.5 μ M of *l*-malate, 0.07 μ M of cytochrome *c*, and varying amounts of cytochrome reductase and pigeon liver enzyme (specific activity at time of use, 100). Final volume, 1.5 cc.; gas, air; temperature, 22°. Corex cells, 0.5 cm. diameter. Wave-length, 550 m μ . Curves 1 to 4, reaction started by addition of 3.75 units of "malic" enzyme; Curve 5, reaction started by addition of 0.75 unit of "malic" enzyme. Cytochrome reductase; Curve 1, 10 γ ; Curve 2, 25 γ ; Curve 3, 50 γ ; Curves 4 and 5, 75 γ . Curve 6, all components but one present at zero time; at 2 minutes, system completed by addition of either 3.75 units of "malic" enzyme ($\blacktriangle$), 75 γ of cytochrome reductase ($\circ$), *l*-malate ($\triangle$), or TPN ($\square$).

FIG. 5. Rate of reduction of cytochrome *c* by *l*-malic acid as a function of the TPN concentration. Samples with 3.75 units of "malic" enzyme, 75 γ of cytochrome reductase, and varying amounts of TPN; otherwise as in Fig. 4. Reaction started by addition of "malic" enzyme.

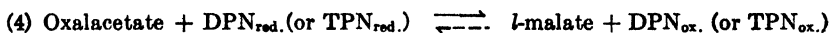
results, illustrated in Table V, clearly show that both "malic" enzyme and lactic dehydrogenase are required.

Reaction with Cytochrome c—The reduction of ferricytochrome *c* by the "malic" enzyme system in the presence of cytochrome reductase (4) is illustrated in Figs. 4 and 5. Fig. 4 shows the effect of the concentration

of reductase and "malic" enzyme on the rate of the reaction (Curves 1 to 5), as well as the indispensability of each of the components of the system (Curve 6). Fig. 5 illustrates the dependence of the reaction rate on the concentration of TPN. The latter data indicate the suitability and high sensitivity of this system for the quantitative assay of TPN, since amounts of TPN as low as 0.02 γ can be accurately and specifically determined. A method of TPN assay by use of the glucose-6-phosphate dehydrogenase-cytochrome reductase-cytochrome *c* system has been described by Haas *et al.* (14). Cytochrome *c* was prepared by the method of Keilin and Hartree (15). The sample of cytochrome reductase was one previously used (16), kindly supplied by Dr. Kurt I. Altman.

DISCUSSION

The results presented in this paper support the view that Reaction 1 is catalyzed either by a single enzyme or by enzymes which are so closely associated as to form a single functional unit. The term "malic" enzyme is used to avoid confusion of this type of enzyme, which catalyzes a reversible *oxidative decarboxylation* of *l*-malic acid by pyridine nucleotide, with malic dehydrogenase, which catalyzes a reversible *oxidation* of *l*-malic acid by pyridine nucleotide (Reaction 4). The TPN-specific "malic" enzyme of pigeon liver does not catalyze the reduction of oxalacetate by



TPN_{red.} within the range pH 4.5 to 7.5 and, therefore, is not a TPN-malic dehydrogenase. In fact, at the optimum pH for catalysis of Reaction 1 (pH 7.5) the "malic" enzyme is neither a malic dehydrogenase nor an oxalacetic carboxylase, since at this pH it also fails to catalyze the decarboxylation of oxalacetate. It may be again emphasized that a mixture of Mn⁺⁺, DPN, or TPN, purified malic dehydrogenase of pig heart, and purified oxalacetic carboxylase of *Micrococcus lysodeikticus*, each enzyme being devoid of "malic" enzyme activity, fails to catalyze Reaction 1 in either direction (1, 3).³ Little interaction between these two enzymes systems would be expected if the intermediate substrate

³ Note added at proof correction. Herbert (20) has recently succeeded in purifying the *M. lysodeikticus* oxalacetic carboxylase (which catalyzes Reaction 2 from left to right) to a very high degree. With a combination of this highly active enzyme, malic dehydrogenase, and DPN he was able to obtain oxidative decarboxylation of malate. This positive result was undoubtedly made possible by the use of large amounts of carboxylase. Nevertheless, under similar conditions he failed to obtain the reverse reaction, i.e. reductive carboxylation of pyruvate, as measured by oxidation of DPN_{red.} in the presence of high concentrations of pyruvate and CO₂ (Society for Experimental Biology, Symposium on carbon dioxide fixation, Sheffield, July 1-5, 1950).

2. The presence of Mn^{++} is indispensable for activity in each of the reactions catalyzed by the "malic" enzyme; Mg^{++} is much less effective than Mn^{++} . The saturation and half saturation values of the protein- Mn^{++} , protein-malate, and protein-oxalacetate complexes have been determined. The affinity of the enzyme for *l*-malic acid is very high.

3. The enzymatic decarboxylation of oxalacetic acid is strongly inhibited by both *d*- and *l*-malate and by malonate.

4. Further evidence is presented in support of the previous finding that the TPN-linked dismutation between *l*-malate and pyruvate, to yield pyruvate, CO_2 , and lactate, is catalyzed by the pigeon liver "malic" enzyme plus lactic dehydrogenase.

5. The reduction of cytochrome *c* by *l*-malic acid, in the presence of "malic" enzyme, Mn^{++} , TPN, and cytochrome reductase, is described.

6. The nature of the "malic" enzyme is discussed in the light of the above and previous findings.

Our thanks are due to Miss Priscilla J. Ortiz for help with some of the experiments and Mr. Morton C. Schneider for help with the enzyme preparation.

The various preparations used were obtained as described in previous papers (1, 3).

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BIOSYNTHESIS OF DICARBOXYLIC ACIDS BY CARBON DIOXIDE FIXATION

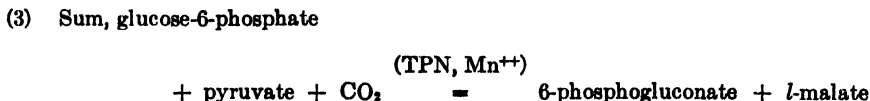
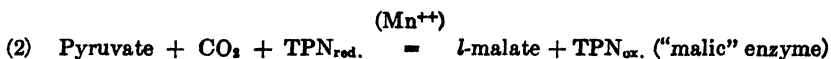
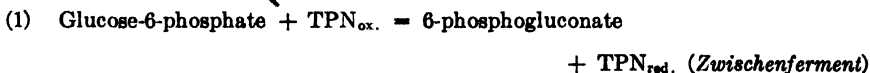
III. ENZYMATIC SYNTHESIS OF L-MALIC ACID BY REDUCTIVE CARBOXYLATION OF PYRUVIC ACID*

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(Received for publication, April 5, 1950)

It has previously been shown (1) that *l*-malate can be formed by a TPN-linked dismutation involving glucose-6-phosphate, pyruvate, and CO₂, in the presence of glucose phosphate dehydrogenase (*Zwischenferment*) and the "malic" enzyme of pigeon liver. Further study of this reaction shows that it proceeds in accordance with the postulated mechanism,¹



A complete chemical balance which demonstrates the occurrence of Reaction 3 has now been obtained. There is no reaction in the absence of either TPN, *Zwischenferment*, or "malic" enzyme. The rate of reaction is proportional to the concentration of "malic" enzyme and to the CO₂ tension.

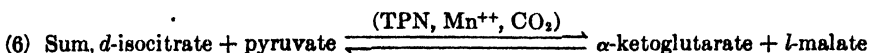
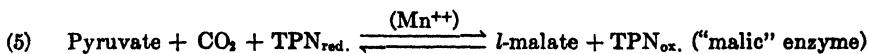
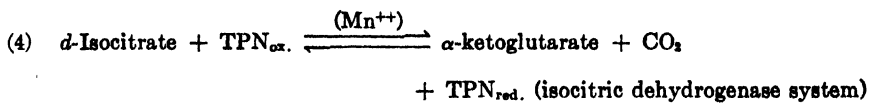
It is also possible to couple the isocitric dehydrogenase-oxalosuccinic carboxylase system (2) with "malic" enzyme and to obtain a reversible TPN-linked dismutation between *d*-isocitrate and pyruvate, or between

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¹ TPN = triphosphopyridine nucleotide; DPN = diphosphopyridine nucleotide.

l-malate and α -ketoglutarate to give *l*-malate or *d*-isocitrate respectively. This dismutation proceeds according to the following reactions.



The equilibrium of Reaction 6 is markedly in favor of malate synthesis.

EXPERIMENTAL

Enzymatic Determination of l-Malate and Fumarate—*l*-Malate can be rapidly and specifically determined with an enzyme preparation from malate-adapted *Lactobacillus arabinosus* (3). This preparation catalyzes the almost quantitative conversion of *l*-malate to lactate and CO_2 . The pH optimum is 6.0. The amount of CO_2 evolved, corrected for the small retention at pH 6.0, is a measure of the amount of *l*-malate present. *d*-Malate does not react. The sensitivity of the determination can be increased about 3-fold by using small Warburg vessels having a capacity between 6 and 7 cc., instead of the conventional ones of about 18 to 20 cc.

The suitability of the method for the purposes of the present work is illustrated in Table I. For the composition of the reaction mixtures, see Table I. The relatively crude enzyme preparation used for this work had a specific activity between 150 and 200 (Korkes *et al.* (4)) and contained a small amount of fumarase. Hence, as shown in Table I, Experiment 4, fumarate caused a protracted evolution of CO_2 . If purified fumarase is added to the reaction mixture, fumarate gives rise to a rapid and practically quantitative evolution of CO_2 , and can therefore be determined quantitatively either alone or together with *l*-malate (Experiments 4 and 5). Highly purified amorphous fumarase was prepared by the method of Laki and Laki (5) (*cf.* also Scott (6)). There was a small evolution of CO_2 in the absence of substrate; this blank was subtracted from the experimental runs. It has always been our practice to run one or two standards with known amounts of fumarate or *l*-malate, simultaneously with the experimental samples. The values obtained in the latter can thus be corrected for the recovery in the standards.

Table I shows that, when present in amounts between 100 and 50 μM , glucose-6-phosphate, pyruvate, glucose, citrate, *dl*-isocitrate, and succinate did not give rise to significant evolution of CO_2 above the blank and did

TABLE I

Determination of Fumarate and l-Malate with *Lactobacillus arabinosus* Enzyme and Fumarase

All values are expressed in micromoles.

Experiment No.	Additions										Dicarboxylic acid (fumarate + malate)	
	Fumarase	l-Malate*	Fumarate	Glucose-6-phosphate	Pyruvate	Glucose	Citrate	dl-Isocitrate	α-Ketoglutarate	Succinate	Found (corrected for blank)	Per cent recovered
1		1.835									0.35†	
		3.67									1.72	93.7
		5.51									3.54	96.5
											4.90	89.0
2				91	105						0.54	
				91	105						0.55	
		3.67									3.36	91.5
3											0.29	
		1.0									0.83	83.0%
		2.0									2.19	109.5
		4.0									3.60	90.0
4†		6.0									5.95	99.0
			6.0								0.16	
	+										1.79	30.0%
	+		6.0								0.16	
5	+										5.96	99.3
	+		3.0								0.25	
	+	2.0	1.8								3.05	102.0
	+	3.0	3.0								3.76	98.9
6											5.97	99.3
		3.0									0.37	
										50	2.95	98.3
		3.0								50	0.34	
7								100			2.93	97.7
								100			0.32	
		3.0									2.92	97.4
		3.0									0.44	
8									50		2.74	91.3
		3.0							50		-0.17	
							50				2.55	85.0%
		3.0					50				0.35	
9											2.73	91.0
		3.0									0.17	
					50						2.82	94.0
		3.0			50						0.32	
10						50					2.80	93.3
		3.0				50					0.24	
11											2.69	89.8
Average												96.9

TABLE I—*Concluded*

0.25 cc. of 2.0 M acetate buffer, pH 6.0, 0.04 cc. of 1.0 M phosphate buffer, pH 6.0, 3.0 μ M of MnCl_2 , and 0.225 μ M of DPN, with other additions as indicated, placed in the main space of small Warburg vessels (capacity, 6 to 7 cc.). Fumarase, when used, was also placed in the main space (about 500 units). Lyophilized *L. arabinosus* enzyme was dissolved in water just before use and an amount of solution, usually containing from 500 to 750 units, was placed in the side bulb. Final volume, 1.5 cc.; gas, air; temperature, 25°. Reaction started by tipping in the bacterial enzyme after temperature equilibration. The reaction is usually complete within 40 to 80 minutes.

* Added as *dl*-malate.

† Blanks expressed as micromoles of CO_2 .

‡ Duration 40 minutes.

§ Not included in the average.

not interfere with the determination of 3 μ M of malate. There was some interference by α -ketoglutarate (Experiment 7). The lower limit of the method is about 2 μ M of *l*-malate (Experiment 3).

Chemical Balance of Dismutation between Glucose-6-phosphate and Pyruvate Plus CO_2 —A highly purified preparation of pigeon liver "malic" enzyme (7) was employed in all the experiments reported in this paper. Crude preparations of *Zwischenferment* were used.² The *Zwischenferment* was contaminated with fumarase; thus a mixture of fumarate and *l*-malate was formed from pyruvate + CO_2 . No attempt was made to determine these two compounds separately.

As in the case of the analogous dismutation between glucose-6-phosphate and α -ketoglutarate + CO_2 (2), Reaction 3 can be followed manometrically when carried out in a medium containing bicarbonate. This is made possible by the fact that, while the absorption of 1 CO_2 equivalent is compensated by liberation from the bicarbonate of another CO_2 equivalent by the second carboxyl group of the newly formed dicarboxylic acid (pK₂ at 25°, *l*-malic acid 5.08, fumaric acid 4.54), the oxidation of glucose-6-phosphate to 6-phosphogluconic acid creates an extra carboxyl group which displaces a CO_2 equivalent.

Two reaction mixtures were set up. The composition of each was as follows: 200 μ M of glucose-6-phosphate, 200 μ M of pyruvate, 300 μ M of NaHCO_3 , 0.54 μ M of TPN, 4 μ M of MnCl_2 , 25 units of *Zwischenferment*, and 260 units of "malic" enzyme. The final volume was 2.0 cc. Sample A served as a zero time control. Immediately after addition of the "malic" enzyme, the sample was acidified with 0.1 cc. of 4.0 N acetic acid, heated at 100° for 3 minutes, and cooled. Sample B was set up in a small Warburg vessel (capacity 6 to 7 cc.) with the "malic" enzyme in the side bulb.

* Some of the *Zwischenferment* used here was kindly supplied by Dr. Erwin Haas some years ago. Another sample was generously given to us by Dr. B. L. Horecker.

After equilibration with 75 per cent CO₂ and 25 per cent N₂, the reaction mixture, the pH of which was about 7.0, was incubated at 25°. The reaction was started by tipping the "malic" enzyme into the main space after temperature equilibration. The reaction was allowed to proceed for 4 hours. During this time, the CO₂ evolution due to the production of acid was measured. As explained above, this is a measure of the production of 6-phosphogluconic acid from glucose-6-phosphate. At the end

TABLE II

Chemical Balance of Dismutation between Glucose-6-Phosphate and Pyruvate + CO₂

Determination of dicarboxylic acid (fumarate + *l*-malate) with *L. arabinosus* enzyme in the presence of fumarase. Conditions as in Table I. Sample A, zero time; Sample B, incubated (see the text).

Time	CO ₂ evolution, c.mm.					
	0.3 cc. Sample A	0.3 cc. Sample A + 5 μ M fumarate	0.2 cc. Sample B	0.3 cc. Sample B	0.1 cc. Sample B	0.1 cc. Sample B + 3 μ M <i>l</i> -malate
<i>min.</i>						
5	2.5	34.2	34.2	38.4	21.3	33.6
10	3.2	64.8	63.0	79.3	37.3	64.7
15	4.4	86.4	82.0	110.0	44.0	88.3
30	5.7	110.5	100.0	147.0	56.2	115.8
55	6.3	116.5	107.0	158.0	57.0	123.0
60	7.0	116.5	108.0	158.5	57.0	123.0
CO ₂ evolution corrected for blank, c.mm.		109.5	101.0	151.5	50.0	66.0
Fumarate + <i>l</i> -malate found, μ M		4.89	4.52	6.77	2.25	2.95
Recovery, %		97.8				98.3
Fumarate + <i>l</i> -malate found, corrected for recovery, μ M			4.61	6.90	2.30	
Total fumarate + <i>l</i> -malate formed, μ M			48.4	48.4	48.3	

of the incubation period, the vessel was cooled in ice and its contents then treated as the first sample. The various determinations were carried out on suitable aliquots of the supernatants obtained by centrifugation. The sum of fumarate and *l*-malate was determined with the *L. arabinosus* enzyme in the presence of purified fumarase, as outlined in the previous section. The experimental details and results are illustrated in Table II. It will be seen that the small evolution of CO₂ obtained with aliquots of Sample A is of the same order of magnitude as that of the blanks of Table I. Pyruvate and glucose-6-phosphate were determined enzymatically, as

outlined below under "Analytical methods," with lactic dehydrogenase and *Zwischenferment* respectively.

The complete balance of the dismutation is shown in Table III. It will be observed that the amount of fumarate + malate formed ($48 \mu\text{M}$ in the 2.0 cc. of reaction mixture, or 24 per cent of the pyruvate present at the beginning) is only about 74 per cent of the amount of phosphogluconic acid produced or of that of glucose-6-phosphate or pyruvate disap-

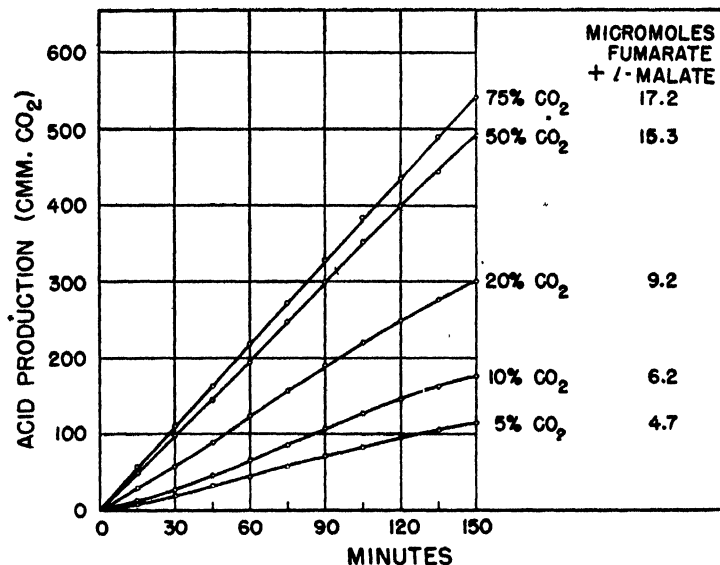


FIG. 2. Rate of dismutation between glucose-6-phosphate and pyruvate + CO_2 , as a function of the tension of CO_2 . All samples contained $100 \mu\text{M}$ of glucose-6-phosphate, $100 \mu\text{M}$ of pyruvate, $0.27 \mu\text{M}$ of TPN, $2 \mu\text{M}$ of MnCl_2 , 12.5 units of *Zwischenferment*, and 150 units of "malic" enzyme. NaHCO_3 , reading from the bottom 10, 20, 40, 100, and 150 μM . Gas, N_2 with the indicated percentages of CO_2 . The pH was 7.0 throughout. Final volume, 1.0 cc.; temperature, 25° . Reaction started by tipping in "malic" enzyme from the side bulb of Warburg vessels after temperature equilibration.

pearing, the latter three magnitudes averaging $65 \mu\text{M}$. This discrepancy is due to interference by the lactic dehydrogenase which contaminates the "malic" enzyme preparation. Under these conditions some glucose-6-phosphate reacts with pyruvate to give phosphogluconate and lactate. It will be remembered that lactic dehydrogenase can react with TPN, although at a much smaller rate than it reacts with DPN (8).

Time Course—The rate of the dismutation as a function of the concentration of "malic" enzyme and the absence of reaction if either

Zwischenferment, "malic" enzyme, or TPN is omitted are shown in Fig. 1. The reaction was followed manometrically, and at the end of the incubation period the amount of *l*-malate + fumarate formed was determined by the enzymatic method. The effect of increasing tensions of CO₂ at constant pH on the rate of dismutation is illustrated in Fig. 2. It will be observed that the reaction proceeds at an appreciable rate, even when the concentration of CO₂ in the gas phase is as low as 5 per cent. This indicates that the system is remarkably effective in bringing about fixation of CO₂.

TABLE IV

Dismutation between Isocitrate and Pyruvate + CO₂ or between Malate and α -Ketoglutarate + CO₂

Each sample contained 150 μ M of NaHCO₃, 0.27 μ M of TPN, 2 μ M of MnCl₂, 150 units of "malic" enzyme, 300 units of isocitric dehydrogenase, and other additions as indicated. Final volume, 1.0 cc. Gas, 75 per cent CO₂ and 25 per cent N₂; pH 7.0; temperature, 25°. Reaction started by tipping in "malic" enzyme from the side bulb of Warburg vessels after temperature equilibration. All values are expressed in micromoles.

Experiment No.	Incubation time	Additions				Δ			
		<i>d</i> -Isocitrate	Pyruvate	<i>l</i> -Malate	α -Ketoglutarate	<i>d</i> -Isocitrate	Pyruvate	Fumarate + <i>l</i> -malate	Acid (manometric)
	<i>min.</i>								
1	210	100	100					+32.3	+5.9
2	210	100	100					+26.0	+5.4
3	210			100	100	+2.6	+3.8		
4	240			100	100	+2.3	+2.6		

Dismutation between d-Isocitrate and Pyruvate Plus CO₂—A consideration of the reactions catalyzed by the isocitric dehydrogenase system and by the "malic" enzyme of pigeon liver, both TPN-specific, suggests that interaction between the two systems will occur to give the reversible Reaction 6. This reaction is a TPN-linked dismutation resulting in the reductive carboxylation of pyruvate at the expense of the oxidative decarboxylation of isocitrate or in the reverse reaction. Typical experiments demonstrating the occurrence of Reaction 6 in either direction are illustrated in Table IV. The isocitric dehydrogenase used was a partially purified preparation (9); it contained some fumarase. *d*-Isocitrate was determined enzymatically with isocitric dehydrogenase (2). Experiments 1 and 2 show the formation of malate when Reaction 6 proceeds from left to right. The small production of acid is probably due to the fact that the third carboxyl of isocitrate (pK_3 of citrate at 25°, 5.4) is replaced by the stronger

second carboxyl of malate and fumarate. Experiments 3 and 4 show the formation of *d*-isocitrate and pyruvate when the reaction proceeds from right to left. No attempts have been made to determine the equilibrium constant of Reaction 6, but it is apparent that this reaction markedly favors the reductive carboxylation of pyruvate.

DISCUSSION

It appears that the "malic" enzyme, when coupled with suitable hydrogen donors, is extremely effective in bringing about CO_2 fixation. CO_2 is first incorporated into *l*-malic acid by the action of the "malic" enzyme (10) and is then distributed in other dicarboxylic acids, such as oxalacetic, fumaric, and succinic, through reactions catalyzed by malic dehydrogenase, fumarase, and succinic dehydrogenase. Recent animal studies (11, 12) demonstrate the remarkable rapidity with which CO_2 finds its way into carbohydrate and other cytoplasmic components via dicarboxylic acids and the reactions of the tricarboxylic and glycolytic cycles. The distribution of the "malic" enzyme in animal cells appears to be less restricted than earlier work led us to believe. The enzyme is present in relatively large amounts in avian liver, but it undoubtedly occurs in other tissues. The isotope studies referred to (11, 12) indicate the presence of the enzyme in mammalian tissues. Extracts of ox brain acetone powder have recently been found in this laboratory³ to contain nearly as much "malic" enzyme as pigeon liver extracts. Its occurrence in mammalian kidney is suggested by the finding of Green *et al.* (13) that pyruvate is rapidly oxidized by washed homogenates in the absence of added dicarboxylic acids, provided that bicarbonate is present. In animal tissues and heterotrophic bacteria the "malic" enzyme is probably concerned with the maintenance and regulation of the level of dicarboxylic acids, thus controlling tissue respiration and metabolism.

The great effectiveness of the "malic" enzyme in CO_2 fixation, however, suggests that enzymes of this type may perform an essential function when CO_2 fixation is really vital; namely, in chemosynthesis and photosynthesis. This view is strengthened by the finding of Vennesland and her associates (14) that a TPN-specific "malic" enzyme is widely distributed in plants. It is gradually becoming apparent that the patterns of glycolysis and respiration in plants closely follow those established for animal cells (15, 16), and it is tempting to speculate that photosynthesis (and chemosynthesis) may operate in a cyclic pattern which might essentially represent the reversal of a respiratory cycle (17). One such cycle, involving a "malic" enzyme, would be a Thunberg-Wieland cycle in reverse, as proposed by Calvin and Benson (18). Such a cycle is illustrated in Fig. 3.

³ J. R. Stern, unpublished results.

CO_2 would be fixed by two mechanisms: (a) by β carboxylation involving "malic" enzyme, and (b) by α carboxylation, involving $\text{C}_2 + \text{C}_1$ condensation to form pyruvate. Occurrence of the latter reaction type in heterotrophic bacteria is established (19, 20), although its mechanism is still obscure (21). A major obstacle for accepting such a cycle, *i.e.* the lack of substantial evidence for the succinate $\rightarrow$ acetate reaction, may be removed by the recent demonstration of the biological occurrence of the conversion of acetate to fumarate by $\text{C}_3 + \text{C}_2$ condensation (22).

The view is gradually gaining ground (23) that the basic mechanisms of CO_2 fixation may be essentially the same in autotrophic and heterotrophic cells, differing only in the way in which energy is supplied and in

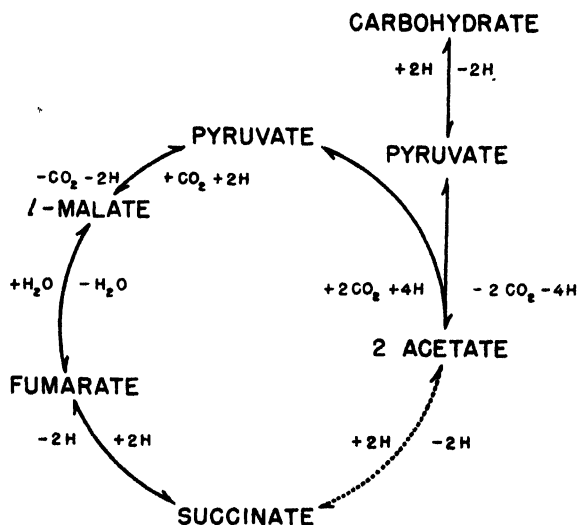


FIG. 3. Thunberg-Wieland cycle

the source of hydrogens for reduction. It is conceivable that the photolytic cleavage of water by illuminated chloroplast preparations might under appropriate conditions substitute for the glucose dehydrogenase or isocitric dehydrogenase systems used in our experiments to bring about the reductive carboxylation of pyruvate.

Analytical Methods

Enzymatic methods were used throughout this work. The determination of *l*-malate and fumarate has already been described. Pyruvic acid was determined spectrophotometrically with lactic dehydrogenase and reduced DPN as in earlier work (1). *d*-Isocitric acid was determined with TPN and partially purified isocitric dehydrogenase (9) as previously

described (2). Glucose-6-phosphate was determined with TPN and *Zwischenferment* (Reaction 1). Under our conditions this determination was specific; i.e., no component in the reaction mixtures other than glucose-6-phosphate reacted. The analytical reaction mixtures, in Corex or silica cells (1.0 cm. in diameter), contained 0.025 M glycylglycine buffer, pH 7.4, 0.135 μ M of TPN, 10 units of *Zwischenferment*, deproteinized unknown solution or known amounts of glucose-6-phosphate containing not over 0.1 μ M of the ester, and water to a final volume of 3.0 cc. The blank cell contained neither TPN nor *Zwischenferment*. Readings of the optical density at wave-length 340 m μ were taken in the Beckman spectrophotometer until there was no further increase; this end-point was reached in about 20 minutes. An optical density of 0.01 corresponds to 0.0048 μ M of the ester. With standard solutions of glucose-6-phosphate it was found that the reaction came practically to a standstill when 80 per cent of the ester had reacted. The results with unknown solutions were therefore corrected for this recovery.

Zwischenferment was assayed optically (Reaction 1) with enough glucose-6-phosphate to saturate the enzyme. Fumarase was assayed in the standard optical test for the pigeon liver "malic" enzyme (7) with fumarate instead of malate as substrate and an excess of "malic" enzyme. There was no reaction in the absence of fumarase. Isocitric dehydrogenase was assayed as previously described (9). 1 enzyme unit was defined in all cases as the amount of enzyme causing an increase in optical density of 0.01 per minute at 23°, calculated for the third 15 second period after the start of the reaction. The *L. arabinosus* "malic" enzyme was assayed as described by Korkes *et al.* (4).

SUMMARY

A study has been made of the biosynthesis of *l*-malic acid by the TPN-linked dismutation between glucose-6-phosphate and pyruvic acid + CO₂ catalyzed by a combination of glucose-6-phosphate dehydrogenase (*Zwischenferment*) and highly purified "malic" enzyme from pigeon liver. In view of the remarkable effectiveness of this system in bringing about CO₂ fixation, the possible rôle of "malic" enzyme in the biological assimilation of CO₂ is discussed.

Our thanks are due to Mr. Morton C. Schneider for skilful technical assistance.

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FUNCTION OF BIOTIN IN THE METABOLISM OF LACTOBACILLUS ARABINOSUS*

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Reports from various laboratories suggested a function of biotin in carbon dioxide fixation (1-7). An enzyme mediating the fixation of CO₂ in dicarboxylic acids was isolated in this laboratory from pigeon liver (8, 9). The activity of this enzyme ("malic" enzyme) was specifically and markedly decreased in the livers of biotin-deficient turkeys (5). Reactivation could not be brought about by biotin or by boiled extracts of either normal liver or purified pigeon liver enzyme. It was further found that highly purified preparations of the pigeon liver "malic" enzyme were essentially free from biotin (5). This suggested that biotin did not function as a prosthetic group in CO₂ fixation enzyme systems, and that its relation to such systems was of a less direct nature.

It was known that some bacteria bring about a dissimilation of malic acid as represented by Reaction 1 (10). It appeared that Reaction 1 might be an over-all reaction involving "malic" enzyme in a partial step, and a search was made for Reaction 1 in bacteria which required biotin as a growth factor.



Resting cells of *Lactobacillus arabinosus* (strain 17-5), when harvested from the usual media with glucose as the main energy source, evolve but little CO₂ from malate. However, when harvested from a growth medium supplemented with malate, the cells catalyze Reaction 1 most vigorously. This activity is markedly decreased in biotin-deficient cells. It appears

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that the greatly increased catalysis of Reaction 1 is due to the formation of an adaptive, DPN-specific "malic" enzyme.¹

The enzyme system can be readily extracted from the adapted cells (11) and has been partially purified. The isolation and properties of the adaptive enzyme are described in the following paper (12). The present paper deals with the formation of the adaptive enzyme and with its relation to biotin. Our results suggest that biotin may be involved in the synthesis of the enzyme.

Results

Reactions Catalyzed

The over-all reaction carried out by non-multiplying washed cells of malate-adapted *L. arabinosus* results in the anaerobic conversion of *l*-malic

TABLE I

Chemical Balance of Dissimilation of Malic Acid by Resting Cells of Malate-Adapted L. arabinosus

Samples contained 0.027 M *l*-malic acid adjusted to pH 4.0, 0.001 M MnCl₂, 0.1 M phosphate, pH 4.0, and water suspension of the organisms. Final volume, 3 cc.; gas, 100 per cent N₂; temperature, 38°. Values expressed in micromoles.

Bacterial N per vessel	Incubation time	Δ malate	Δ lactate	Δ CO ₂
mg.	min.			
0.163	43	-45	+42	+43
0.282	60	-53	+56	+52

acid to lactic acid and CO₂. Evidence for this is presented in Table I. *d*-Malic acid is not attacked.

Organisms grown in the usual media ferment glucose, 2 molecules of lactic acid being produced for each glucose molecule disappearing. They will also rapidly dissimilate pyruvic acid, with production of carbon dioxide. Although this reaction has not been studied in detail, it appears to be a dismutation reaction whereby 2 molecules of pyruvic acid give rise to 1 molecule each of lactic acid, acetic acid, and carbon dioxide. A small amount of fumarase is found in both intact cells and in extracts of the cells. The organisms do not appear to contain malic dehydrogenase.

All of these reactions are catalyzed by both biotin-deficient and normal organisms. In all cases, however, the reaction rate with deficient organisms is slower than with normal organisms.

¹ DNP = diphosphopyridine nucleotide; TNP = triphosphopyridine nucleotide.

Adaptive Nature of Malic Acid Dissimilation System

Adaptation of L. arabinosus during Growth—Although the enzyme system which converts malic acid to lactic acid and carbon dioxide is present in organisms harvested from the Wright and Skeggs growth medium (in which glucose serves as the main energy source), its activity is extremely low. However, when malic acid is also present during growth, the malate dissimilation activity of the bacteria is markedly increased. Table II (Experiments 1 and 2) illustrates comparative results with organisms cul-

TABLE II

Adaptation and Loss of Adaptation of L. arabinosus 17-5, to Malic Acid during Growth

Experimental details as described in the text. Glycolysis samples contained 0.037 M glucose, 0.0016 M $MgCl_2$, 0.02 M $NaHCO_3$, 0.003 M phosphate, pH 7.4, and a suspension of washed organisms. Final volume, 3.0 cc.; gas, 95 per cent N_2 and 5 per cent CO_2 ; temperature, 38°. Malate and pyruvate samples contained 0.022 M substrate, 0.0016 $MnCl_2$, 0.1 M phosphate, pH 4.0, and a suspension of washed organisms. Final volume, 3.0 cc.; gas, N_2 ; temperature, 38°.

Experiment No.	Growth substrate	Q_{CO_2} (N)*		
		Glycolysis	Malate dissimilation	Pyruvate dissimilation
1	Glucose		66	
2	" + malate		9321	
3	"	2000	42	2500
4	" + pyruvate	1600	40	1000
5	" + malate	1200	2000	250
6	"		41	
7	" + malate		3600	

* C.mm. of CO_2 evolved per hour and mg. of bacterial nitrogen.

tured in the Wright and Skeggs medium, without and with the addition of 2 per cent *dl*-malic acid.

Formation of an adaptive enzyme rather than selection of a mutant is indicated by the rapidity with which the activity is decreased upon returning the organisms to a growth medium containing no malic acid. Normal organisms were cultured in glucose or in glucose and malate. After 48 hours of growth the organisms from each culture were harvested and their rates of glycolysis and malic acid dissimilation compared (Table II, Experiments 3 and 5). By addition of malic acid to the growth medium, the activity toward malic acid was increased about 50 times. Just before harvesting, equal aliquots of suspensions of the organisms grown in glucose and malate (Experiment 5) were removed and subcultured in glu-

cose (Experiment 6) and glucose plus malate (Experiment 7). After 24 hours of growth these cultures were harvested and the activity toward malate determined. That of the organisms which had been transferred from a glucose-malate to a glucose medium had returned completely to the unadapted level. The organisms that had undergone a second sub-culture in a glucose-malate medium had increased their activity toward malate above the level of those which had been cultured only once in a malic acid-containing medium.

While the malate activity of the organisms is increased during the process of adaptation to malic acid, there is a decreased capacity of the malate-adapted organisms to dissimilate pyruvic acid (Table II, Experiments 3 and 5). Also culturing the organisms in pyruvate does not increase their activity toward this substrate (Experiment 4).

TABLE III

Activity of Certain Enzymes in Extracts of Malate-Adapted and Unadapted Organisms

Experimental details as described in the text. Values expressed as specific activity (enzyme units per mg. of protein). Temperature, 25°.

Condition of organisms	"Malic" enzyme	Oxalacetic carboxylase	Fumarase	Lactic dehydrogenase
Unadapted	0	0	0.013	140
"	0.5	0.5	0.13*	250
Adapted	40	49	0.16	212
"	50	52	2.8*	290

* Extract incubated for 30 minutes with fumarate before test.

Extraction of Adaptive Enzyme—To determine whether the increased malate activity of the adapted organisms was the result of adaptation in the sense that it involved a greater amount of enzyme protein formed, the activities of the malate dissimilation system in extracts of adapted and unadapted organisms were compared. The effect of adaptation to malic acid upon the activity of lactic dehydrogenase, which was presumably not involved in the adaptive process, was also determined. The results are illustrated in Table III.

Acetone powders of both types of cells were extracted by grinding with 0.02 M phosphate, pH 7.0, for 3 hours in a mechanical mortar in the cold room. After centrifugation the "malic" enzyme and oxalacetic carboxylase activities in the supernatants were determined as described in the following paper (12). The lactic dehydrogenase test has been described elsewhere (13). Fumarase activity was determined by spectrophotometric measurement of the rate of reduction of TPN by fumaric acid in the pres-

ence of highly purified preparations of "malic" enzyme from pigeon liver. The sensitivity of the fumarase test was increased by incubating fumarate with the bacterial extract for 30 minutes before adding the pigeon liver enzyme and TPN. 1 unit was defined as the amount of enzyme causing an increase of optical density of 0.01 per minute.

The "malic" enzyme and oxalacetic carboxylase activities in the ex-

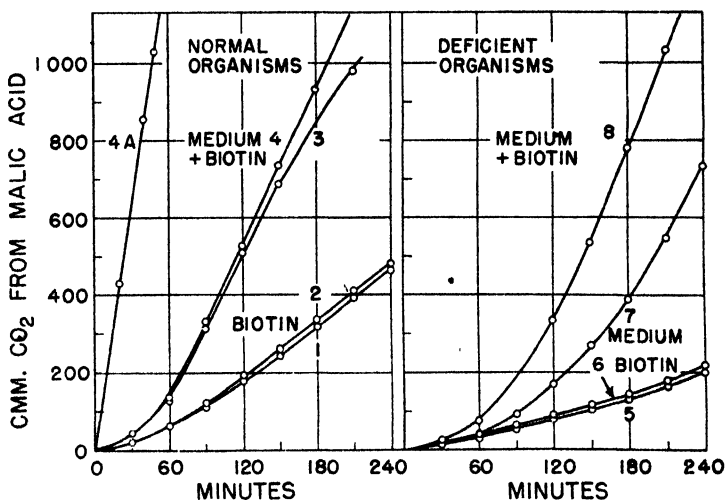


FIG. 1. Adaptation of non-multiplying cells to malic acid. Experimental conditions as described in the text. The samples contained 0.04 M glucose, 0.08 M *DL*-malic acid, pH 5.5, 0.002 M $MgCl_2$, 0.002 M $MnCl_2$, 0.02 M phosphate, pH 5.5, suspension of washed organisms, and other additions as indicated. Four vessels (Curves 1 to 4) contained normal organisms, 0.213 mg. of N per vessel. Four vessels (Curves 5 to 8) contained biotin-deficient organisms, 0.200 mg. of N per vessel. Other additions, 0.5 μ gm. of biotin (Curves 2, 4, 6, and 8) and 0.2 cc. of Wright and Skeggs double strength medium without glucose or biotin (Curves 3, 4, 7, and 8). Final volume, 2.5 cc. One vessel (Curve 4A) contained malate-adapted normal organisms (0.165 mg. of N), 0.027 M *L*-malic acid, 0.1 M phosphate, pH 4.0, and 0.002 M $MnCl_2$. Final volume 3.0 cc.; gas N_2 ; temperature, 38°.

tracts of adapted organisms were both increased approximately 100 times (Table III). On the other hand, the specific activity of lactic dehydrogenase, which is probably a constitutive enzyme, was about the same in the extracts of adapted and unadapted cells. Although the activity of fumarase is very low in extracts from both types of organisms, adaptation to malate raised it to some extent.

Adaptation in Non-Multiplying Organisms—Resting cells of *L. arabinosus* are capable of being adapted to malic acid. The organisms were grown on the Wright and Skeggs medium to which glucose, but not malic acid,

had been added, either in the presence or absence of biotin; these unadapted cells were used for the experiments illustrated in Fig. 1. The experiments were carried out at pH 5.5, in the presence of glucose as a source of energy; glycolysis at this pH amounted to 75 to 80 per cent of that at pH 7.0. The malate dissimilation activity of both types of organisms was followed manometrically by measuring the rate of production of carbon dioxide from *l*-malic acid. Little or no growth occurred during the experimental period as judged by turbidity measurements of the bacterial suspensions before and after incubation.

The occurrence of adaptation is indicated by the autocatalytic nature of the curves. Curve 4A, obtained with previously adapted organisms, is shown for comparison. In the presence of glucose, the cells of normal organisms required only the addition of medium for adaptation (Fig. 1, Curves 3 and 4). In the case of the biotin-deficient organisms, addition of both biotin and medium was essential (Curves 7 and 8).

Relation of Biotin to Malic Acid Dissimilation System

Malate Activity of Adapted Normal and Biotin-Deficient Organisms—The malate activity of biotin-deficient organisms was consistently found to be much lower than that of the normal ones and addition of biotin was without effect. This is illustrated in Table IV. Despite maintenance in each experiment of this sharp difference of activity, considerable variation of the level of activity of both deficient and normal organisms was observed in different experiments performed on different days. No single factor to which this variability may be attributed was found.

The metabolism of biotin-deficient organisms is rather generally impaired, since these organisms showed some depression of both the rate of glycolysis and that of pyruvate dissimilation. In a number of experiments the decrease in malate dissimilation of biotin-deficient organisms averaged 65 per cent, while the decrease in glycolysis averaged 34 per cent.

Deficiency of either pantothenic acid or pyridoxine did not result in decreased malate dissimilation of the resting cells.

Activation of Biotin-Deficient Organisms—A slow reactivation of the malate dissimilation system of biotin-deficient cells takes place when biotin is added together with a small amount of growth medium containing glucose. This is illustrated in Fig. 2. There is little or no bacterial growth over a 4 hour period. As illustrated in Table V, the presence of glucose is needed for this activation by medium and biotin. The biotin-deficient cells were incubated for 90 minutes in the presence of Mg^{++} , Mn^{++} , and phosphate, pH 7.4, with other additions as indicated. At the end of this period the pH was brought to 4.0 and, upon addition of malate, the rate of CO_2 evolution was measured.

In order to determine which components of the medium were effective in activating, together with glucose and biotin, the malate system of biotin-deficient cells, the following experiments were carried out. Biotin-deficient organisms were incubated with glucose and biotin in the presence or absence of several combinations of the components of the medium. The latter had the same concentration in the mixtures as in an equivalent amount of medium. After allowing the cells to glycolyze at pH 7.0 for 60 to 90 minutes, the pH was reduced to 4.0 and the evolution of CO_2

TABLE IV

Malate Dissimilation in Normal and Biotin-Deficient Malate-Adapted Organisms

Samples contained 0.1 M phosphate, 0.0016 M MnCl_2 , 0.027 M l-malate (adjusted with sodium hydroxide to the desired pH), and a suspension of organisms in water (0.1 to 0.2 mg. of N), with other additions as indicated, in a final volume of 3.0 cc. Reaction started by tipping in organisms from the side bulb after temperature equilibration. Gas, N_2 ; temperature, 38°. Values expressed as Q_{CO_2} (N) calculated on the rate during the 1st hour, except in Experiment 5 in which the rate was calculated on the first 30 minutes.

Experiment No.	No. of washings of cells after harvesting	Initial pH	Q_{CO_2} (N)			
			Normal organisms		Biotin-deficient	
			Biotin added		Biotin added	
			None	0.5 γ	None	0.5 γ
1	3	4	5880	5740	2560	2560
	3	5	3500	3528	2990	2767
2	4	4	6300	6400	212	257
	4	5	3670	3510	1535	1480
3	4	4	3810		138	
	4	5	3150		420	
4	4	4	3854		1404	
5	1	4	9321		5590	
6	1	4	8600		3300	
	2	4	7500		1020	

from malate was measured. As illustrated in Table VI, the activation by the medium is mainly due to the amino acids contained in it.

Since DPN is the coenzyme of the "malic" enzyme of *L. arabinosus* (12), it seemed of interest to determine whether nicotinic acid deficiency would affect malate dissimilation and whether addition of nicotinic acid would restore the activity. Two experiments were carried out simultaneously (Fig. 3). In the first, malate activity of adapted nicotinic acid-deficient organisms was tested with and without the addition of nicotinic acid (Curves 1 and 2, respectively). In the second, malate activity of

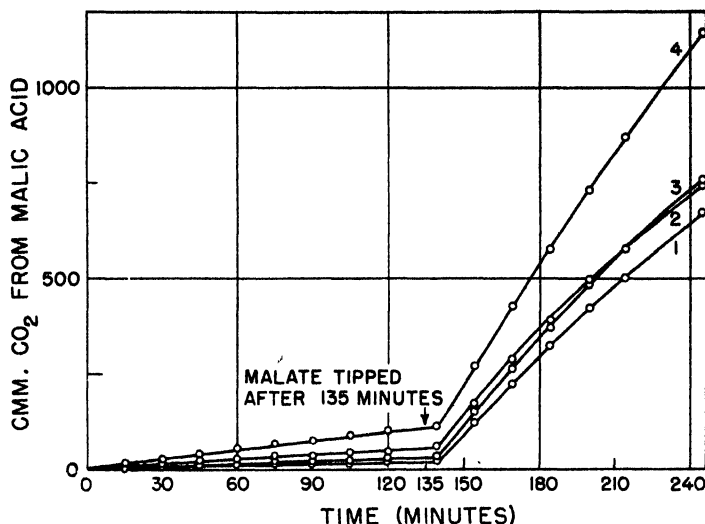


FIG. 2. Activation of malic acid dissimilation system after a preliminary period of glycolysis at pH 5.5 in the presence of medium and biotin. All vessels contained 0.08 M *dl*-malic acid, pH 5.5, in the side arm and, in the main space, 0.04 M glucose, 0.002 M MnCl_2 , 0.002 M MgCl_2 , 0.02 M phosphate, pH 5.5, and 0.3 cc. of a suspension of deficient organisms containing 0.273 mg. of N. 0.5 γ of biotin was also added in Vessels 2 and 4; 0.2 cc. of Wright and Skeggs' double strength medium in Vessels 3 and 4. Malic acid was tipped into all vessels after 135 minutes. Final volume, 2.5 cc.; gas, N_2 ; temperature, 38°.

TABLE V

Need of Glucose for Activation of Malate Dissimilation in Biotin-Deficient Organisms

For details see the text. Q_{CO_2} (N) values calculated for the 2nd hour after addition of malate.

Additions	(N)	
	Glucose absent	Glucose (100 μM) present
None.....	3260	2839
Biotin (0.5 γ)	3182	3162
Medium* (0.2 cc.)	2946	3026
" and biotin	2221	4053

* Without glucose or biotin.

adapted biotin-deficient organisms was determined in the presence and absence of added biotin (Curves 3 and 4, respectively). Since the synthesis of DPN would require energy, glucose was added to all vessels.

TABLE VI

Increase in Malate Activity of Biotin-Deficient Organisms Following Period of Glycolysis in Presence of Amino Acids and Biotin

Experimental details as described in the text. Experiments 1 and 3, addition of 0.2 cc. of double strength medium or its equivalent in amino acids, bases, or vitamins. Experiment 2, addition of an amount of amino acids equivalent to 0.3 cc. of double strength medium. All rate values were calculated on the 1st hour of reaction.

Experiment No.	Normal cells	Biotin-deficient cells		Per cent increase of Q_{CO_2} (N) of biotin-deficient cells upon addition of							
	Q_{CO_2} (N)	Q_{CO_2} (N)	Per cent of normal	Biotin*	Medium	Biotin, medium	Biotin, bases, vitamins	Biotin, amino acids, bases	Biotin, amino acids, vitamins	Amino acids	Biotin, amino acids
1	6100	2330	38.2	15.0	18.8	54.0	18.7	49.7	52.9		
2	6300	2290	36.3	15.2						7.4	45.2
3	6425	1120	17.4	12.0	11.6	56.2					41.0

* 0.5 γ per flask.

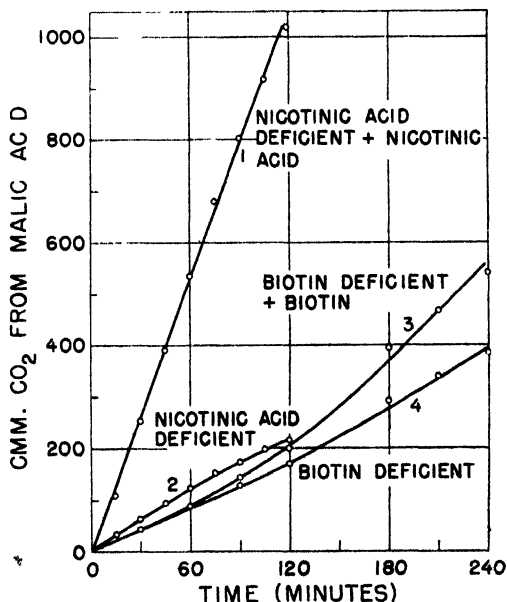


FIG. 3. Reactivation of malate dissimilation system of nicotinic acid and biotin-deficient cells. All samples contained 0.04 M glucose, 0.08 M *dl*-malic acid, pH 5.5, 0.002 M $MgCl_2$, 0.002 M $MnCl_2$, 0.02 M phosphate, pH 5.5, and 0.2 cc. medium without glucose, nicotinic acid, or biotin, with other additions as indicated (nicotinic acid, 0.2 γ ; biotin, 0.5 γ). Final volume, 2.0 cc.; gas, N_2 ; temperature, 38°.

Hughes and Williamson (14) have recently reported a rapid synthesis of DPN on addition of nicotinic acid to nicotinic acid-deficient cells of *L. arabinosus* 17-5; in the absence of glucose the synthesis was negligible (see also McIlwain *et al.* (15)). In our experiments the removal of nicotinic acid from the growth medium resulted in a marked decrease of DPN in the organisms from 2 to 3 γ per mg. of bacterial nitrogen to almost nil. This was shown by analyzing samples of the nicotinic acid-deficient and normal organisms for DPN.

Fig. 3 shows that in the case of the nicotinic acid-deficient organisms, in which the rate of dissimilation of malate is also markedly decreased, addition of nicotinic acid resulted in an immediate stimulation and attainment of maximal rates (Curves 1 and 2), whereas in the case of the biotin-deficient organisms the addition of biotin was followed by a long induction period before any stimulation of malate dissimilation occurred (Curves 3 and 4). The stimulatory effect of nicotinic acid may be assumed to be due to the capacity of the nicotinic acid-deficient organisms to synthesize DPN readily when nicotinic acid is available, and it is likely that other prosthetic groups would be synthesized at a comparable rate. Since the biotin activation is very slow, it appears more likely that biotin participates not in the synthesis of a prosthetic group but in that of some other component of the system which is formed more slowly, perhaps the enzyme protein itself.

Determination of Dicarboxylic Acids with L. arabinosus

Non-multiplying or lyophilized cells of *L. arabinosus* can be used for the quantitative determination of *l*-malic acid by measurement of the CO_2 evolution. Typical results are shown in Table VII.

In Experiments 1 and 2 (Table VII) the organisms were grown for 48 hours in the Wright and Skeggs medium, supplemented with biotin and malic acid. In the others the organisms were incubated for 24 hours in 2 per cent glucose, 2 per cent *dl*-malic acid, and 2 per cent yeast extract (Difco), or in 2 per cent glucose, 2 per cent *dl*-malic acid, 1 per cent yeast extract, 1 per cent nutrient broth, 1 per cent sodium acetate- $3\text{H}_2\text{O}$, and 0.1 per cent K_2HPO_4 with the addition of 0.5 γ of biotin and 0.5 cc. of Salts B of the Wright and Skeggs medium (16) per 100 cc. Washing the organisms is not necessary, since their endogenous release of CO_2 is negligible. An amount of organisms representing as little as 0.3 mg. of N per vessel can catalyze the quantitative evolution of CO_2 from 10 μM of malic acid within 30 minutes. Lyophilized organisms can also be used. An amount of aqueous suspension containing 20 mg. of lyophilized organisms per vessel gives satisfactory results. Such estimations are illustrated in Experiments 4, 5, and 6, Table VII.

It may be seen that, in the amounts used, L-aspartate does not interfere with the malate determination (Experiments 2 and 3). Fumaric acid is slowly transformed to malic acid by the small amount of fumarase present in the organisms. If fumaric acid is known to be present in a sample to be assayed for malic acid, it is advisable to add fumarase and

TABLE VII

Determination of Dicarboxylic Acids with L. arabinosus

All samples contained 0.1 M phosphate, 0.0016 M MnCl₂, and a suspension of either intact cells (Experiments 1, 2, and 3), representing 0.27, 0.44, and 1.27 mg. of N, respectively, or lyophilized organisms (20 mg. per vessel). Other additions as indicated. Final volume, 3.0 cc.; gas, 95 per cent N₂ and 5 per cent CO₂; temperature, 38°. Organisms tipped in from the side bulb after temperature equilibration. Unless otherwise indicated, the evolution of CO₂ was completed within 30 minutes. Substrates and phosphate adjusted to the pH indicated.

Experiment No.	Cell preparation	Initial pH	Additions	CO ₂ evolved
			μM^*	μM
1	Intact	4	None	0.10
			<i>l</i> -Malate (8)	8.13
2	"	4	None	0
			<i>L</i> -Aspartate (10)	0
			" (10), <i>l</i> -malate (8)	8.15
3	"	5	" (10)	0
			<i>l</i> -Malate (10)	9.4
			<i>L</i> -Aspartate (10), <i>l</i> -malate (10)	9.5
			<i>l</i> -Malate (10), pyruvate (50)	17.8†
			" (10), " (50), semicarbazide (50)	9.8
4	Lyophilized	5	<i>l</i> -Malate (10)	9.7
5	"	5	" (10)	9.4
6	"	5	" (10)	9.8
7	"	6	" (10), fumarate (5), fumarase	14.2
8	"	6	Fumarate (10), fumarase	9.7
9	"	5	<i>l</i> -Malate (10), pyruvate (40), semicarbazide (44)	9.7

* Figures in parentheses.

† Gas evolution going on steadily after 30 minutes.

determine the sum of malic and fumaric acids. These estimations were carried out at pH 6.0, which is close to the optimum pH for fumarase (Experiments 7 and 8). Pyruvic acid is rapidly dissimilated both by fresh and lyophilized cells. Therefore, if present, the reaction will continue until both malic and pyruvic acids have been consumed (Experiment

3). This difficulty may be circumvented by adding semicarbazide to bind the pyruvic acid. Semicarbazide, brought to pH 4.0 or 5.0, is added in equimolecular quantity or in a small excess of the amount of pyruvic acid present. In this way, with either intact or lyophilized organisms, malic acid can be determined in the presence of pyruvic acid (Experiments 3 and 9).

A similar method for determining *l*-malic acid with *Bacterium gracile* has been developed by Rentschler (17).

DISCUSSION

The results of the study of the malic acid dissimilation system in *L. arabinosus* support the view that biotin is not involved in it as a prosthetic group or a component of such a group. The comparison of the reactivation of malate activity by nicotinic acid (a component of DPN which participates in malate dissimilation) and biotin may be mentioned in this connection. In both nicotinic acid and biotin deficiency the activity is markedly decreased. However, addition of nicotinic acid to nicotinic acid-deficient cells incubated with glucose and medium produced an immediate reactivation of malate activity, while addition of biotin to biotin-deficient cells, under otherwise identical conditions, resulted in extremely slow reactivation. This indicates that synthesis of DPN takes place easily (14). Rapid synthesis of a complex prosthetic group is not unprecedented. Novelli and Lipmann (18) found that non-multiplying pantothenic acid-deficient cells of *Proteus morganii* rapidly synthesized coenzyme A. These results, together with earlier ones which demonstrated that the specific decrease of "malic" enzyme activity in the liver of biotin-deficient turkeys cannot be explained as a deficiency of a biotin-containing prosthetic group (5), suggest that biotin is not incorporated into a prosthetic group required by the malate dissimilation system.

The hypothesis that biotin might function in carbon dioxide fixation by exchanging carbon dioxide through alternate opening and closing of the ureido ring (19) was recently tested by Melville *et al.* (20) with biotin containing C^{14} in the ureido carbon atom. When *L. arabinosus* was cultured in an aspartic acid-free medium containing radioactive biotin, there was no appreciable dilution of the radioactivity, indicating that such a mechanism was inoperative.

Olson *et al.* (21) have also found that biotin activation cannot be readily shown *in vitro*. Pyruvate and succinate oxidation and $C^{14}O_2$ production from $C^{14}OOH$ succinate in heart ventricle slices from biotin-deficient ducks were lower than that of normal controls. Attempts to raise the depressed respiration of the slices by incubating the slices with biotin for 30 minutes before adding the substrates failed to produce a significant increase. How-

ever, if the birds received an injection of biotin, respiration was restored to the normal range within 24 hours.

Only minute concentrations of biotin are required for bacterial growth. This substance is present in various tissues at very low concentrations relative to other vitamins which function as components of prosthetic groups of certain enzyme systems of intermediary metabolism (*i.e.* nicotinic acid, thiamine, pyridoxine, pantothenic acid). These facts make it unlikely that biotin is involved as a prosthetic group at the level of metabolism of the malic acid dissimilation system. It would appear that biotin functions at a more basic level and that it may be a component of the prosthetic group of an enzyme system which synthesizes (or is involved in the synthesis of) other prosthetic groups or apoenzymes. The latter possibility is made more likely in our experiments because of the fact that combination of biotin, the amino acids of the medium, and glycolytic energy will slowly reactivate the malic acid dissimilation system. The need of biotin for the adaptive formation of the "malic" enzyme also supports this view.

Since biotin deficiency affected glycolysis and pyruvate dissimilation, and biotin appears to be involved in the function of other enzymes (22), it might be involved in synthesis of protein in a general way. Its relatively larger effect upon the malate system may be merely a reflection of a high rate of turnover (synthesis and destruction) of the "malic" enzyme. This would be particularly so in the case of *L. arabinosus* in which this enzyme is adaptive.

Our results with *L. arabinosus* are comparable to those of Winzler *et al.* (23) with biotin-deficient yeast. *Saccharomyces cerevisiae* can synthesize its own amino acids and requires only ammonia as a source of nitrogen. Stimulation of fermentation did not occur when biotin alone was added, although it was quickly absorbed and incorporated into the deficient yeast cells. However, if ammonia was added along with the biotin, slow, prolonged stimulation occurred. Analogously, in our experiments stimulation of glycolysis and malate activity in *L. arabinosus* is dependent upon both biotin and the amino acids of the medium.

Methods

Bacteriological—*L. arabinosus* 17-5 was carried on 1.5 per cent agar, 1 per cent yeast extract, and 1 per cent glucose slants, transfers being made once a month. The inoculum used for growing large batches of organisms for an experiment was prepared by transfer from the stock culture to 10 cc. of a liquid medium containing 1 per cent glucose and 1 per cent yeast extract. After incubation for 18 hours at 30°, this culture was used to inoculate flasks of medium in the proportion of 1 cc. of inoculum per 100 cc. of medium. The synthetic culture medium was that described by

Wright and Skeggs (16) with the addition, unless otherwise specified, of 2 per cent *dl*-malic acid. Biotin either was omitted from the medium or was added to a final concentration of 0.25 to 0.33 μ gm. per cc. When biotin was added to the medium, excellent growth was obtained. The total yield of bacterial nitrogen from 75 cc. of biotin-containing medium was about 40 mg. When no biotin was added, only meager growth occurred; consequently, it was necessary to inoculate 400 to 500 cc. of medium. From this amount of medium, after 48 hours of growth, the bacterial population represented only 2 to 3 mg. of total cell nitrogen.

The organisms were harvested by centrifugation, washed with cold water, and suspended in ice-cold water; an amount of water was used such that the turbidity of the suspension as determined in a Klett-Summerson photoelectric colorimeter, with a No. 660 filter, was equivalent to about 1 mg. of total cell nitrogen per cc. By comparison with a previously determined standard curve, which related turbidity to total nitrogen of a suspension, the amount of organisms placed in each Warburg vessel was adjusted to contain 0.1 to 0.3 mg. of N.

Manometric—For the manometric experiments chilled solutions were added to the vessels, which were kept in an ice bath. All the additions were made to the main chamber, except the washed, iced suspension of the organisms, which was placed in the side arm. Intact cells of *L. arabinosus* are quickly inactivated by exposure to phosphate at pH 4.0. The presence of malate, glucose, or medium protects the organisms against this inactivation. For this reason the suspension of organisms was always tipped into the reaction mixture after temperature equilibration. The vessels were gassed with 95 per cent nitrogen and 5 per cent carbon dioxide or with nitrogen while being cooled in ice, and then placed in the bath at 38°. The reaction was started by tipping in the bacterial suspension from the side arm after temperature equilibration. Values expressed as Q_{CO_2} (N) indicate c.mm. of CO_2 evolved per hour and mg. of bacterial nitrogen.

Analytical—Analyses were performed on neutralized trichloroacetic acid filtrates of the reaction mixtures, except for glucose which was determined by the method of Shaffer and Somogyi (24) on zinc sulfate-barium hydroxide filtrates (25). Lactic acid was determined by the method of Barker and Summerson (26). *l*-Malic acid was determined enzymatically with purified "malic" enzyme of pigeon liver as previously described (8).

SUMMARY

1. An extremely active "malic" enzyme is present in *Lactobacillus arabinosus* after culturing the organism in the presence of malic acid. The enzyme is an adaptive one, and its formation beyond the constitutive

level may be induced during growth in the presence of malic acid, or may be developed in non-multiplying cells on incubation with malic acid. Normal organisms require glucose and medium for adaptation, whereas biotin-deficient organisms require biotin in addition to glucose and medium. The adaptive process involves synthesis of the "malic" enzyme; extracts of adapted cells contain about 50 times more "malic" enzyme than those of non-adapted cells.

2. Biotin-deficient malate-adapted *L. arabinosus* shows in comparison to normal organisms a marked decrease of activity of the malate dissimilation system and a less marked decrease both of the rate of glycolysis and of pyruvate dissimilation.

3. Partial reactivation of the malate dissimilation system of biotin-deficient organisms occurs on incubation with both biotin and a small amount of growth medium or its component amino acids. This reactivation, which requires glycolytic energy, proceeds very slowly, in contrast to reactivation with nicotinic acid of nicotinic acid-deficient organisms, which, under otherwise identical conditions, proceeds very rapidly.

4. The results of previous and of the present work suggest that the relationship of biotin to enzyme systems of CO₂ fixation is less direct than that of a prosthetic group or a component of a prosthetic group. It appears that biotin may be involved in some manner in the synthesis of these and possibly of other enzymes.

5. A method is outlined for the manometric determination of *l*-malic acid with fresh or lyophilized cell suspensions of malate-adapted *L. arabinosus*.

We wish to express our appreciation to Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company, for his interest in this work, for kindly supplying a number of components of the synthetic growth medium, and for biotin assays carried out in his laboratory. Our thanks are also due to Dr. J. A. Aeschlimann of the Research Laboratories, Hoffmann-La Roche, Inc., for a generous supply of *d*-biotin and for samples of imidazolidonecaproic acid and *dl*-homobiotin used in some preliminary experiments. We are also indebted to Dr. H. Lüthi, Agriculture Experiment Station, Wädenswil, Switzerland, for cultures of the malate-fermenting organism *Bacterium gracile*, and to Dr. E. Racker for a culture of a malate-fermenting organism isolated by him; these organisms were investigated in preliminary experiments.

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BIOSYNTHESIS OF DICARBOXYLIC ACIDS BY CARBON DIOXIDE FIXATION

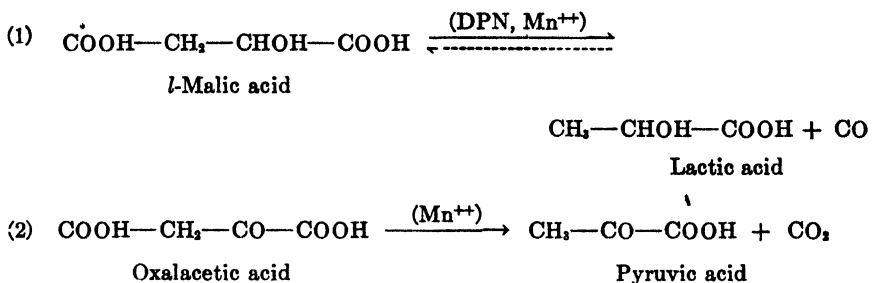
IV. ISOLATION AND PROPERTIES OF AN ADAPTIVE "MALIC" ENZYME FROM LACTOBACILLUS ARABINOSUS*

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Lactobacillus arabinosus (strain 17-5) may be adapted to ferment *l*-malic acid (1). As previously reported (2), the enzyme system can be readily extracted from the organism and has been partially purified. The partially purified enzyme catalyzes Reactions 1 and 2.¹

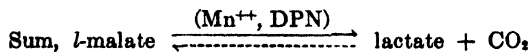


Reaction 1 requires DPN and manganous ions. It is specific for *l*-malic acid; *d*-malic acid is inactive. TPN cannot replace DPN. Reaction 2 requires the presence of manganous ions. The evidence presented below supports the view that Reaction 1 is a DPN-linked dismutation between *l*-malate and pyruvate, resulting from the interaction of lactic dehydrogenase with a DPN-specific "malic" enzyme as shown by Reactions 3 and 4.



* Aided by grants from the United States Public Health Service, the American Cancer Society (recommended by the Committee on Growth of the National Research Council), the Office of Naval Research, and the Lederle Laboratories Division, American Cyanamid Company.

¹ Solid line arrows indicate the direction of faster reaction. The usual abbreviations are used: DPN for diphosphopyridine nucleotide, TPN for triphosphopyridine nucleotide, and ATP for adenosinetriphosphate.



The "malic" enzyme of *L. arabinosus* appears to be entirely similar to the "malic" enzyme of pigeon liver (3) except that it is DPN- rather than TPN-dependent. The reversibility of Reaction 1 has been demonstrated with C^{14}O_2 , but its equilibrium position is overwhelmingly in favor of decarboxylation. The reaction can be utilized for the quantitative determination of *l*-malate (1, 4).

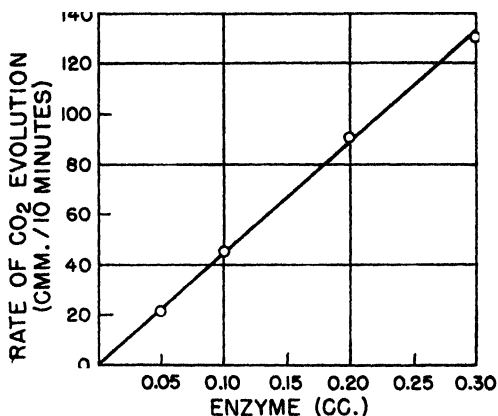


FIG. 1. Rate of CO_2 evolution from *l*-malate as a function of the enzyme concentration. Standard test system. Enzyme, extract of acetone powder (specific activity 90) containing 5 mg. of protein per cc.

EXPERIMENTAL

Manometric Test and Enzyme Unit—The rate of liberation of CO_2 from *l*-malate in the presence of DPN and Mn^{++} (Reaction 1) is proportional to the enzyme concentration, as illustrated in Fig. 1. The reaction mixture consists of 0.08 M phosphate buffer, pH 6.0, 0.002 M MnCl_2 , 1.2×10^{-4} M DPN, 0.06 M *l*-malate, pH 6.0, enzyme, and water. An excess of lactic dehydrogenase was also added to the reaction mixture in case the concentration of this enzyme in the bacterial preparations should become limiting on purification. This has so far not been the case. Final volume, 1.0 cc.; gas, air; temperature, 25°. The reaction is carried out in small Warburg vessels of about 6 cc. capacity, and is started after temperature equilibration by tipping the enzyme from the side bulb. Readings are taken at 5 minute intervals. Under these conditions the reaction is kinetically of zero order as long as the enzyme is saturated with substrate.

The volume of CO_2 produced is calculated by correcting the vessel constant for the small CO_2 retention at pH 6.0.

The test for oxalacetic carboxylase activity (Reaction 2) is carried out in the same way. The reaction mixture contains 0.08 M acetate buffer, pH 4.5, 0.08 M phosphate adjusted to the same pH, 0.002 M MnCl_2 , 0.023 M oxalacetate (acid dissolved just before use and adjusted to pH 4.5), enzyme, and water. In this case a blank without enzyme is run simultaneously to correct for the spontaneous decarboxylation of oxalacetate. The evolution of CO_2 due to the enzyme is of zero order over a good part of its course and is proportional to the enzyme concentration. 1 enzyme unit is arbitrarily defined in both tests as the amount of enzyme catalyzing the liberation of 1.0 c.mm. of CO_2 in 10 minutes, calculated for the second 5 minutes following tipping in the enzyme. The amount of enzyme is adjusted so that the rate of CO_2 liberation is between 30 and 90 c.mm. per 10 minutes.

The protein content of the enzyme solution is determined by either of two methods. In the initial stages, when the nucleic acid content is high, protein is estimated by the method of Heidelberger and MacPherson (5) as modified by Lowry.² In the later stages, protein is determined spectrophotometrically by measurement of the light absorption at wavelengths 280 and 260 $\text{m}\mu$. A correction for the nucleic acid content, from the data given by Warburg and Christian (6), is used to calculate the protein concentration. The specific activity of the enzyme is expressed as units per mg. of protein.

Isolation of Enzyme

L. arabinosus 17-5 is the starting material. The organisms are grown on a non-synthetic medium containing glucose and *l*-malate (1). After 24 hours, the cells are harvested by centrifugation and suspended in distilled iced water to make a thin paste.

Preparation of Acetone Powder—The chilled bacterial paste is poured all at once into 10 volumes of acetone chilled to -10° in a Waring blender; the mixture is homogenized for 2 minutes and then filtered by suction in the cold room. The cake is washed twice with small portions of cold acetone and the suction is continued until the cake is almost dry. The residue is then crumbled by hand, spread on filter paper, and allowed to dry in the air at room temperature. The dry material is ground to a fine powder in a mechanical mortar and stored in stoppered bottles in the ice box. The yield of dry powder is about 3 gm. per liter of growth medium. When stored in the cold, the powder is stable for at least 1 year.

² Personal communication. We are indebted to Dr. O. H. Lowry for this information.

The following steps are carried out in the cold.

Step 1. Extraction—3 gm. of powder are extracted with 150 cc. of 0.02 M phosphate buffer, pH 7, by grinding the mixture in a mechanical mortar for 3 hours at 3–4°. The insoluble residue is removed by centrifugation at 0° and discarded. The viscous extract (about 130 cc.) contains 4 to 5 mg. of protein per cc. Specific activity 80 to 90.

Step 2. First Ammonium Sulfate Fractionation—To 130 cc. of extract are added 45 gm. of solid ammonium sulfate (degree of saturation 0.5) with mechanical stirring. After 20 minutes, the precipitate is centrifuged at 15,000 r.p.m. and discarded. To the supernatant is added sufficient ammonium sulfate to saturate the solution, and after 30 minutes the mixture is centrifuged and the supernatant discarded. The precipitate is dissolved in 0.02 M phosphate buffer, pH 7.0, and dialyzed against the same buffer overnight. The resultant solution of enzyme (about 30 cc.) contains 7 to 8 mg. of protein per cc. Specific activity about 150.

Step 3. Precipitation of Nucleic Acid and Inactive Protein with Protamine—30 cc. of enzyme solution from Step 2 are brought to pH 6.0 with approximately 0.2 cc. of 1.0 N acetic acid. To this solution is slowly added with mechanical stirring 0.85 cc. of a solution of protamine sulfate (20 mg. per cc.; pH 5.0) per 100 mg. of protein. This quantity is sufficient to bring the ratio of light absorption at 280 m μ to light absorption at 260 m μ of the enzyme solution from values of about 0.5 to 1.0 or slightly higher, indicating a reduction of the nucleic acid content to about 3 per cent, with a minimal precipitation of enzyme protein. The precipitate is centrifuged and discarded. The supernatant (32 cc.) contains approximately 3 mg. of protein per cc. Specific activity about 280.

Step 4. Removal of Inactive Proteins with Calcium Phosphate Gel—The enzyme solution from Step 3 is adjusted to pH 5.5 with 2 drops of 1.0 N acetic acid, and calcium phosphate gel (36 mg. of Ca₃PO₄ per cc.) is added slowly with mechanical stirring in the proportion of 4 cc. per 100 mg. of protein. After 20 minutes the mixture is centrifuged and the precipitate discarded. The supernatant (32 cc.) contains the enzyme. The protein concentration is about 1.0 mg. per cc. Specific activity about 600.

Step 5. Second Ammonium Sulfate Fractionation—The procedure of Step 2 is repeated; the solution is first brought to 0.5 saturation with ammonium sulfate, the precipitate discarded, more ammonium sulfate added to saturation, and the second precipitate suspended in 0.02 M phosphate buffer, pH 7.0. The solution is dialyzed overnight against the same buffer. Volume, 9 cc.; protein about 2.5 mg. per cc.; specific activity about 950.

Step 6. Adsorption on Calcium Phosphate Gel—2.0 cc. of the enzyme solution from Step 5 were mixed with 0.5 cc. of calcium phosphate gel

after being adjusted to pH 5.5 with acetic acid. After stirring for 20 minutes, the mixture was centrifuged and the supernatant discarded. The precipitate was washed with 2 cc. of 0.1 M phosphate buffer, pH 5.5, for 10 minutes and centrifuged. The supernatant contained about one-fourth of the enzyme units at specific activity 850. The precipitate was eluted with 2 cc. of 0.1 M phosphate buffer, pH 6.0, under stirring for 20 minutes. The eluate contained about 0.7 mg. of protein per cc. Specific activity about 1800.

Table I summarizes the results of two typical runs with the procedure described above. It will be seen that the purification is fairly reproduc-

TABLE I
Purification of L. arabinosus Enzyme

3 gm. of acetone powder (a and b separate runs).

Step	Run	Volume of solution	Units	Protein	Specific activity	Yield
		cc.		mg.	units per mg. protein	per cent
Phosphate extract	a	129	52,000	580	89	100
	b	134	54,500	627	87	100
1st ammonium sulfate fractionation	a	31	34,100	217	157	66
	b	31	34,000	258	132	62
Protamine supernatant	a	32	25,100	91	275	48
	b	31	28,000	100	280	51
Calcium phosphate supernatant	a	33	25,000	38	660	48
	b	32	26,400	41	643	48
2nd ammonium sulfate fractionation	a	8.5	24,300	25	988	47
	b	10.4	25,000	26	964	46
Elution from calcium phosphate gel	a*	2.0	2,570	1.4	1840	21

* Aliquot of the previous step.

ble. At various steps in the purification the oxalacetic carboxylase (Reaction 2) and lactic dehydrogenase (Reaction 4) activities of the preparations were measured, and the results expressed as specific activities in Table II. The lactic dehydrogenase test and units were those described in a previous publication (7). It will be noted that over a range of 30-fold purification the ratio of activities with malate and oxalacetate as substrates remains constant, while the ratio of lactic dehydrogenase to "malic" activity falls from about 5 to 1, although lactic dehydrogenase is also partially purified. Although some fumarase is present in the initial extract, as determined by substituting fumarate for malate in the test system, it disappears after the first calcium phosphate treatment.

Properties of Enzyme System

Formation of Lactate—The balance illustrated in Table III indicates that for each mole of malate disappearing 1 mole each of lactate and carbon dioxide is formed, in accordance with Reaction 1. The enzyme is

TABLE II
Ratios of Activities at Various Purification Stages

Specific activity			Ratios	
"Malic" enzyme (E)	Oxalacetic carboxylase (O)	Lactic dehydrogenase (L)	$\frac{O}{E}$	$\frac{L}{E}$
52		288		5.5
87	80	310	0.92	3.6
314	335	943	1.07	3.0
456	492	1190	1.08	2.6
515	506	1220	0.98	2.4
956	915		0.96	
1840		1755		0.95
2620*	2700	3370	1.03	1.3
238†	223	1675	0.94	7.0

* Results obtained by Dr. S. E. Kaufman in the course of further purification of the enzyme.

† After heating a sample of specific activity 414 to 49° for 8 minutes.

TABLE III
Chemical Balance of Reaction 1

Enzyme (1.0 cc. of extract of acetone powder containing 4.7 mg. of protein per cc.) incubated with 150 μ M of *l*-malate, pH 6.0, 0.08 M acetate buffer, pH 6.0, 0.08 M phosphate buffer, pH 6.0, 0.33 μ M of DPN, and 5 μ M of MnCl₂. Final volume, 2.7 cc.; gas, air; temperature, 25°; incubation time, 100 minutes. Analyses performed on aliquots of trichloroacetic acid filtrates as described under "Methods." The values are expressed in micromoles.

	<i>l</i> -Malate	Lactate	CO ₂	Pyruvate
Initial.....	148.0	1.8		0
Final.....	80.5	68.0	60.8	0.14
Δ	-67.5	+66.2	+60.8	+0.14

quite specific for *l*-malate; *d*-malate is not attacked, nor does it inhibit the reaction. It should be noticed that a small amount of pyruvate is formed.

pH Optimum—Fig. 2 represents the pH-activity curves for the reaction with both malate and oxalacetate; *i.e.*, for Reactions 1 and 2. Activity with malate shows a sharp optimum at pH 6.0, whereas activity with oxalacetate exhibits an equally sharp optimum at pH about 4.5. It should

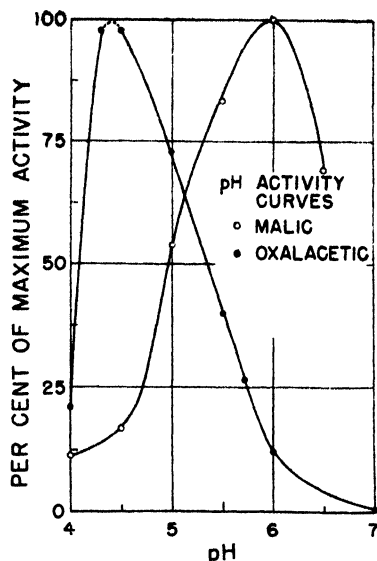


FIG. 2. pH-activity curves. ●, oxalacetic carboxylase activity (Reaction 2); ○, "malic" activity (Reaction 1). Standard manometric tests as described in text. Enzyme of specific activity 150. All samples were buffered with acetate and phosphate adjusted to the desired pH; the substrates were also adjusted to the pH of the measurement. The pH at the end of each run was at the most 0.1 to 0.2 unit higher than the initial value.

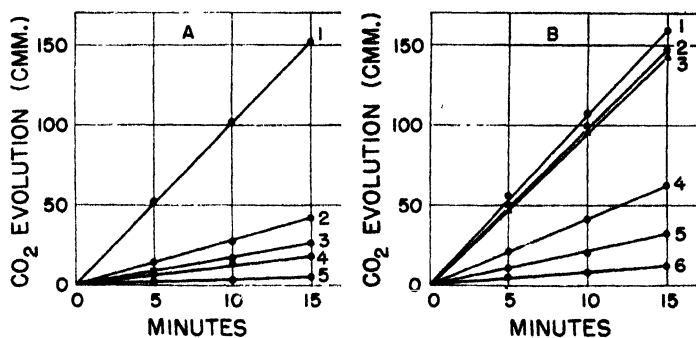


FIG. 3. Components of enzyme system. Enzyme of specific activity 900, dialyzed overnight at 2° against 0.05 M potassium chloride; 0.11 mg. of protein per sample. Standard manometric tests. A, system of Reaction 1, Curve 1, complete system; Curve 2, no phosphate (buffered with succinate); Curve 3, no Mn^{++} ; Curve 4, either no DPN or TPN in place of DPN; Curve 5, no malate. B, system of Reaction 2, Curve 1, complete system; Curve 2, no phosphate (buffered with acetate only); Curve 3, complete plus 1.2×10^{-4} M DPN; Curve 4, complete plus 0.05 M l-malate of pH 4.5; Curve 5, complete plus 0.05 M malonate of pH 4.5; Curve 6, no Mn^{++} . The first 5 minutes after the start of the reaction have been disregarded. All values in B are corrected by subtracting the CO_2 liberated from oxalacetate in the absence of enzyme.

be noted that at pH 6.0 the rate of decarboxylation of oxalacetate is only about one-eighth of the maximal rate, a point which will be discussed below.

Components—In addition to *l*-malate, the system of Reaction 1 requires catalytic amounts of DPN and Mn^{++} ; this is illustrated in Fig. 3, A. It is of interest that, in contrast to the pigeon liver "malic" enzyme, TPN is completely without effect. Co^{++} may replace Mn^{++} completely, but Mg^{++} is considerably less effective. The dissociation constants of the complex between the protein and each of the other components of Reaction 1 are as follows: *l*-malate, 9.8×10^{-3} ; DPN, 1.96×10^{-5} ; Mn^{++} , 2.9×10^{-4} (all expressed in moles $\times$ liter $^{-1}$). In determining the above constants for each of the components all others were present in substantial excess. As illustrated in Fig. 3, B the system of Reaction 2 requires only Mn^{++} in addition to oxalacetate. The dissociation constant of the oxalacetate-enzyme complex is about 10^{-2} (moles of oxalacetate per liter); i.e., essentially the same as that of the *l*-malate-enzyme complex. The half saturation concentration of Mn^{++} in Reaction 2 is about 3×10^{-4} M and is thus identical to the corresponding value for Reaction 1. It is of interest to compare these data with those obtained with pigeon liver "malic" enzyme (8). Included in Fig. 3, B are curves showing the inhibitory action of *l*-malate and malonate on Reaction 2. 0.05 M malate, i.e. about the same concentration at which it is used as the substrate of Reaction 1, caused 60 per cent inhibition of Reaction 2. The same concentration of malonate produced a stronger inhibition (8). Reaction 2 was completely inhibited by 0.12 M malonate, while Reaction 1 was inhibited only to the extent of 30 per cent.

Whereas, with dialyzed acetone powder extracts, Reaction 1 had been found to proceed about equally well in the absence or presence of orthophosphate (2), an activation by phosphate is shown with the purer fractions (Fig. 3, A). This is so in spite of adequate buffering with succinate buffer. In the absence of orthophosphate, activity drops to about one-fourth of that in its presence. The effect of phosphate does not seem to be due to preserving the enzyme from inactivation because activity can be restored to maximal levels on adding phosphate as late as 20 minutes after the reaction is started. Activation by phosphate is only observed in Reaction 1. As shown in Fig. 3, B, removal of phosphate results in no appreciable change in the activity of Reaction 2. Arsenate in low concentrations does not replace the phosphate requirement and in higher concentrations is inhibitory. The mode of action of phosphate remains so far unexplained.³ It does not seem to be involved in phosphorylation reactions;

³ Note added at proof correction. The activation appears to be due to the cation potassium and not to the phosphate ion (potassium phosphate was the buffer used). When potassium succinate is substituted for the sodium succinate, formerly used as

no coupled phosphorylation was observed when Reaction 1 was allowed to proceed in the presence of phosphate, glucose, hexokinase, and catalytic amounts of ATP. ATP is without effect on either Reaction 1 or 2.

Reversibility of Reaction 1—With air in the gas phase, Reaction 1 goes essentially to completion. However, Fig. 4 indicates that the presence of pyruvate with increasing concentrations of CO_2 in the gas phase exerts an increasingly inhibitory effect on the rate of the reaction. No inhibition was observed with lactate instead of pyruvate. This indicated reversibil-

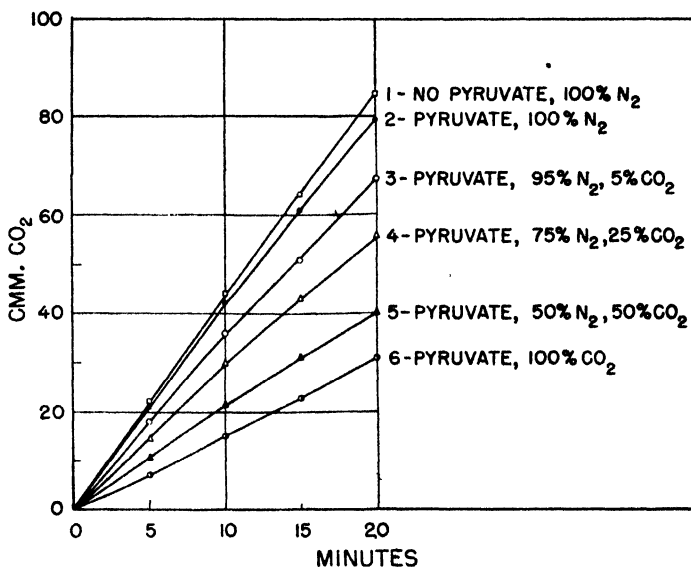


FIG. 4. Inhibition of malate dissimilation by pyruvate and CO_2 . Standard manometric test, except that 0.022 M, instead of 0.06 M, *l*-malate was used. 0.12 M pyruvate was added when indicated. Sufficient NaHCO_3 to give pH 6.0 when equilibrated with each of the gas mixtures was added when CO_2 was present. Enzyme of specific activity 150 with 0.4 mg. of protein in each sample. Final volume, 1.5 cc.

ity of the postulated partial Reaction 3. Evidence for reversibility is presented in Table IV, in which incorporation of isotopic CO_2 in the dicarboxylic acids was measured.

In Experiments 2 and 3, fumarate was substituted for malate and, besides the bacterial enzyme, purified fumarase was added. Fumarase was

buffer in the absence of phosphate, the system was found to be quite active. The effect of K^+ on malate dissimilation by cell suspensions of adapted *L. arabinosus* was discovered by P. M. Nossal in the laboratory of H. A. Krebs. We are indebted to Dr. Nossal and Dr. Krebs for this information. An activating effect of K^+ on a similar system in cell suspensions of *Moraxella lwoffii* had been described earlier by Lwoff and Ionesco (19).

TABLE IV
Reversibility of Malate Dissimilation Catalyzed by L. arabinosus Enzyme

All samples contained 0.08 M phosphate, pH 6.0, 0.002 M MnCl₂, and 1.2×10^{-4} M DPN. Final volume, 6.0 cc. Reactions carried out at 25°.

Experi- ment No.	Additions							Gas phase	Extent of reaction	Total C ¹⁴ fixed		C ¹⁴ in β -carboxyl of dicarboxylic acids	
	Enzyme		Fumarase units	L-Mal- ate μ M	Fuma- rate μ M	Pyr- uvate μ M	C ¹⁴ O ₂ c.p.m.			c.p.m.	per cent	c.p.m.	per cent total fixed
	units	specific activity											
1	1500	150	10,000	300	300	300	500,000	Air	83	5,360	3,390	63	
2	1125	150	10,000		300	300	375,000	100% CO ₂	81	19,600	16,800	86	
3	740	255	10,000		300	300	288,000	None	13	5,700	3,850	67	
4	740	255	20,000	420	150	250	288,000	"	24	13,500	11,740	87	
5*	Pigeon liver extract 3 cc.				250	250	121,900	95% N ₂ -5% CO ₂	Not meas- ured	17,900	5,120	29	

* Experiment carried out under the conditions described by Wood *et al.* (9). The figures in the last two columns of this experiment represent the β -carboxyl of malate only, since the sample was decarboxylated with fumarase-free *L. arabinosus* enzyme without addition of fumarase.

also added in Experiment 4. This was done with the hope of increasing the total incorporation of isotopic CO_2 . Under these conditions, the following reactions occur.



Establishment of the fumarase-catalyzed equilibrium would result both in an appreciable shift of the equilibrium position of the system in the direction of CO_2 incorporation and in a distribution of the isotopic CO_2 primarily fixed in the β -carboxyl group of malate, in both carboxyl groups of the dicarboxylic acids and in the carboxyl groups of lactate and pyruvate.

In one experiment dialyzed pigeon liver extract was substituted for the bacterial enzyme for comparison. This experiment was carried out under conditions similar to those of Wood *et al.* (9). The fixation of CO_2 is greater than with the bacterial enzyme. This may be due to the fact that coupling of Reaction 3 with Reaction 4, which markedly shifts the equilibrium in the direction of decarboxylation, is less efficient in the liver system because of the TPN specificity of its malic enzyme and of the slow rate at which lactic dehydrogenase reacts with TPN (7).

No fixation of CO_2 in oxalacetate was found when the preparations were allowed to decarboxylate oxalacetate in the presence of C^{14}O_2 irrespective of the presence or absence of ATP.⁴ These experiments were carried out by a procedure similar to that described by Utter and Wood (10) and Vennesland *et al.* (11) with pigeon liver enzymes.

Optical Nature of Lactate Formed—With intact cells adapted to malate, the product of glycolysis and of malate dissimilation has been shown to be *dl*-lactate. Crude preparations of the enzyme (specific activity about 100) also form *dl*-lactate from *l*-malate. This is interesting in the light of the strict optical specificity of the enzyme for *l*-malate as substrate. For identification of the lactate the reaction was allowed to proceed to completion; the lactic acid was extracted with ether and isolated as the zinc salt. This proved to be zinc *dl*-lactate on the basis of the water of crystallization, as shown by the following data: per cent H_2O found, 18.09; theory for *dl*-zinc lactate, 18.17; theory for either *d*- or *l*-zinc lactate, 12.90. Polarimetrically, the substance was non-rotatory, both as the zinc salt in neutral solution and as the molybdate complex. The formation of *dl*-lactate is of some interest. Christensen *et al.* (12) have described an enzyme of *Clostridium butylicum* which catalyzes the racemization of either *d*- or *l*-lactic acid. The formation of *dl*-lactate by the *L. arabinosus* preparations might be due to contamination with a racemizing enzyme or to formation of racemic lactate from pyruvate (Reaction 4) by the bacterial lactic dehydrogenase.

⁴ Harary, I., Veiga Salles, J. B., and Ochoa, S., unpublished results.

DISCUSSION

It is hardly necessary to point out that Reaction 1 cannot be a direct decarboxylation of malate to lactate and CO_2 . The requirement for DPN indicates the occurrence of an oxidation-reduction. In the light of previous work (3, 7) it is apparent that such an oxidation-reduction must involve Reactions 3 and 4. Direct spectrophotometric demonstration of Reaction 3 has proved so far impossible, since the purified preparations still contain lactic dehydrogenase, and hence the reduced DPN formed by Reaction 3 is immediately reoxidized by Reaction 4.

An oxidation-reduction similar to the one proposed above has been shown to occur by interaction of the "malic" enzyme of pigeon liver with lactic dehydrogenase (7, 8). Owing to the TPN specificity of the pigeon liver enzyme, this oxidation-reduction is TPN-dependent and its occurrence is possible because lactic dehydrogenase can react with TPN, though only at 1/100 to 1/150 of the rate at which it reacts with DPN. In this case too, the net result of the TPN-linked dismutation is the conversion of *l*-malate to lactate and CO_2 (7, 13).

It would thus appear that the preparations of *L. arabinosus* here described contain a malic enzyme which, except for its DPN (instead of TPN) specificity, is analogous to that of pigeon liver. In both cases, as far as can be determined, the two activities exhibited by each preparation, *i.e.* catalysis of the reversible oxidative decarboxylation of *l*-malic acid and of the decarboxylation of oxalacetic acid, seem to be associated with one protein or functional protein unit. This is evidenced by constant ratios at various levels of purity (see Table II). It should also be recalled that in *L. arabinosus* both activities appear simultaneously on adaptation to malate (1).

The DPN specificity of the bacterial malic enzyme, indicated by the DPN specificity of the overall reaction (Reaction 1), makes its interaction with lactic dehydrogenase extremely rapid and efficient. However, a small amount of pyruvate accumulates, as shown in Table III. This is further evidence that Reaction 1 is the combined result of Reactions 3 and 4.

There is little doubt that in the bacterial preparations lactic dehydrogenase has no relation to the "malic" enzyme other than that of a contaminant, since, as shown in Table II, the ratio of activity of the former to the latter enzyme can be markedly diminished on purification. Moreover, while it is possible to reduce the malate and oxalacetate activities proportionately by progressive heat denaturation, the lactic dehydrogenase activity is affected to a far lesser degree (Table II). As pointed out in the preceding paper (1), lactic dehydrogenase can be demonstrated as a constitutive enzyme in *L. arabinosus* and the total quantities extracted from unadapted and malate-adapted organisms are about the same.

It has been pointed out elsewhere (3, 8) that the activity of the "malic" enzyme cannot be attributed to the combined activities of a malic dehydrogenase and an oxalacetic carboxylase. This is true of both the "malic" enzyme of pigeon liver and that of *L. arabinosus* (8). In the case of the bacterial enzyme, malic dehydrogenase cannot be detected at any stage of purification. The optical test for malic dehydrogenase, based on the reaction $\text{oxalacetate} + \text{DPN}_{\text{red.}} \rightleftharpoons l\text{-malate} + \text{DPN}_{\text{ox.}}$ (7), is highly sensitive and valid under our conditions, since the "malic" enzyme in the preparation will not catalyze Reaction 3, i.e. will not reduce $\text{DPN}_{\text{ox.}}$, in the absence of Mn^{++} . In addition, it has been shown that, while the rate of disappearance of malate at pH 6.0 is equal to that of oxalacetate at pH 4.5 (Table II), at pH 6.0 the rate of oxalacetate decarboxylation is only about one-eighth of the rate of malate dissimilation (see Fig. 2). If two separate enzymatic steps were involved with free oxalacetate as an intermediate, the rate of disappearance of malate could never be higher than that of oxalacetate. Also malonate at concentrations at which it strongly inhibits oxalacetate decarboxylation has little or no effect on malate dissimilation by the bacterial enzyme.

Methods

Preparations—Highly purified amorphous fumarase was prepared according to Laki and Laki (14). An ammonium sulfate fraction from rabbit muscle, kindly supplied by Dr. E. Racker, was used as a source of lactic dehydrogenase. The following substances were prepared as previously described: DPN (7), TPN (15), *d*-malic acid (3). Calcium phosphate gel was prepared as described by Keilin and Hartree (16). *dl*- and *l*-malic acids were obtained commercially. Protamine sulfate was generously supplied by E. R. Squibb and Sons. $\text{Na}_2\text{C}^{14}\text{O}_3$ was obtained on allocation from the United States Atomic Energy Commission.

Analytical—Lactic acid was determined by the method of Barker and Summerson (17). *l*-Malic and pyruvic acids were determined enzymatically, with purified pigeon liver "malic" enzyme and lactic dehydrogenase respectively, as previously described (3).

Isotope Experiments—The reaction mixture was similar to that in the manometric tests. The reaction was carried out in a closed system and, at the end of the experimental period, sulfuric acid was tipped in to give 1.0 N final concentration. The CO_2 was collected in a solution of sodium hydroxide. The sample was alternately flushed with CO_2 and air until the rinses were free of radioactivity. The precipitated protein was centrifuged and the supernatant neutralized. A 0.1 cc. aliquot (Sample 1) was plated and its radioactivity measured in order to determine the total fixation of C^{14}O_2 . Another aliquot (Sample 2) was used for the determination of residual fumaric and malic acids with the *L. arabinosus* enzyme plus

fumarase (4). An aliquot identical to Sample 2 but larger (Sample 3) was run simultaneously with it in a larger vessel containing 1.0 N NaOH in the center well and 10.0 N H₂SO₄ in the side bulb. Incubation of this sample was continued for 30 minutes after evolution of CO₂ in Sample 2 had ceased. The acid was tipped in and the vessel was shaken for another 30 minutes to allow for complete absorption of the CO₂. This CO₂ is liberated from the β -carboxyl of malate and one of the fumarate carboxyls. The alkali in the center well was diluted as required and 0.1 cc. aliquots were plated directly, dried under an infra-red lamp, and counted immediately to minimize water absorption. A thin window Geiger-Müller counter was used. All counts were corrected to zero thickness by the method of Libby (18).

SUMMARY

The isolation, partial purification, and characterization of an adaptive enzyme of *Lactobacillus arabinosus*, catalyzing the oxidative decarboxylation of *l*-malic acid, are described. The enzyme also catalyzes the decarboxylation of oxalacetic acid. Both activities seem to be associated with a single protein or functional protein unit. Owing to the presence of lactic dehydrogenase as a contaminant in the preparations, the over-all reaction with malate as substrate is l -malate $\rightleftharpoons$ lactate + CO₂. This reaction, which has been demonstrated to be reversible by the use of isotopic carbon dioxide, appears to be the result of interaction between "malic" enzyme, catalyzing the oxidative decarboxylation of *l*-malate to pyruvate and CO₂ in the presence of DPN and Mn⁺⁺, and lactic dehydrogenase, catalyzing the reduction of the pyruvate to lactate.

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